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THE CALCIUM, INORGANIC PHOSPHORUS AND MAGNESIUM CONTENT OF THE BLOOD SERUM OF YOUNG HORSES

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(Received for publication February 7, 1940)

The normal ranges of serum calcium, inorganic phosphorus, and magnesium have been reported for mature horses, but such information for young animals is very meager. Pearson ('34) has shown a high negative correlation between age and the inorganic phosphorus content of the serum. The youngest animals studied by him were approximately 6 months of age. The normal range usually given for the calcium and inorganic phosphorus content of the blood serum of mature horses lies between 12 to 13 mg. % and 3 to 4 mg. % respectively. Dimock and Healy ('33) report an average of 12 mg. of calcium and 4.9 mg. of inorganic phosphorus per 100 cc. of blood serum obtained from four thoroughbred yearling fillies. Errington ('37) reports the inorganic phosphorus in the blood sera of ninety-eight horses, 4 years of age and over, and shows a range of 2.56 to 4.83 mg. per 100 cc., mean 3.55 $\pm$ 0.045 mg.; for seven colts 2 to 3 years of age, the range is 4.37 to 5.98 mg., mean 5.13 $\pm$ 0.226 mg.; eight colts 23 to 43 weeks of age gave a range of 4.02 to 5.78 mg., mean 4.95 $\pm$ 0.216 mg. The serum calcium of the same groups of animals, respectively, ranged as follows: 11.50 to 13.70 mg., mean 12.67 $\pm$ 0.048 mg.; 12.10 to 13.50 mg., mean 12.95 $\pm$ 0.177 mg.; and 11.87 to 14.12 mg.,

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mean 12.81 ± 0.290 mg. Pearson and Catchpole ('36) report normal calcium values of 13.01, 13.59 and 13.11 mg. % for Percherons, Arabians and Shetland ponies, respectively. Eveleth ('37) made analyses of magnesium in the blood and reported a range of between 2 to 3 mg. per 100 cc. of serum.

All the foals born in 1936 and 1937 at the University of California Farm were used as experimental animals in our studies. The 1936 foals ranged from 3 to 6 months of age when the first blood samples were taken in August of that year. Samples were taken at intervals until December, 1937. Blood samples were secured on the day of foaling from both the mare and foal in 1937. Subsequent samples were taken from the foal at intervals until December when the experiment was terminated.

In view of the small number of experimental animals employed and the fact that the values obtained from the chemical analyses overlap and are indistinguishable, it was thought best not to segregate the results either as to breed or sex. However, of the nineteen young animals used, five were Thoroughbreds and the remaining fourteen were Percherons.

Approximately 25 cc. of whole blood were drawn from the jugular vein. The samples were allowed to clot and the clear serum was obtained by centrifugation. The calcium was determined by the Clark-Collip ('25) modification of the Kramer-Tisdall method, and the inorganic phosphorus by the method of Fiske and Subbarow ('25). The magnesium determinations were made according to the method of Briggs ('24).

The mean values of the analyses for each age group, together with their standard errors, are presented in the table. The serum calcium, inorganic phosphorus, and magnesium content of the mares at foaling are within the normal range reported for mature horses. Since the foals present more varied results, each of the constituents will be discussed singly. The magnesium content of the blood serum of the foals is not different from the normal range which has been reported for mature horses. The mean values rise from 2.3 ± 0.10 mg. % at birth to 3.1 ± 0.24 mg. at 9 weeks of age; from then on the

magnesium values are fairly constant hovering around 3.0 mg. per 100 cc. of serum. The greatest difference, 1 mg., is found between the mean value at birth and the mean value for the group of animals ranging from 22 to 25 weeks of age. Apparently the magnesium content of the serum changes little with age. So long as the animal is healthy and receives normal feed the magnesium content of the blood serum remains at a fairly constant level.

TABLE 1

Calcium, inorganic phosphorus, and magnesium content of the blood serum

AGE OF ANIMALS	NO. OF ANIMALS	MILLIGRAMS PER 100 CC. BLOOD SERUM		
		Calcium	Inorganic phosphorus	Magnesium
		Mean	*Mean*	*Mean*
Mare at foaling	10	12.7±0.22	3.3±0.19	2.7±0.18
Birth	10	12.5±0.19	5.1±0.15	2.3±0.10
2 to 4 weeks	9	12.8±0.17	7.7±0.19	2.4±0.11
5 to 7 weeks	6	13.1±0.30	7.6±0.31	2.5±0.06
8 to 10 weeks	3	13.2±0.30	7.9±0.33	3.1±0.24
11 to 13 weeks	5	13.4±0.21	7.4±0.13	2.9±0.11
14 to 16 weeks	4	13.2±0.21	7.4±0.42	3.0±0.16
17 to 19 weeks	3	13.3±0.44	7.1±0.25	3.0±0.22
22 to 25 weeks	4	12.7±0.09	7.0±0.20	3.3±0.13
6 to 12 months	23 [1]	12.2±0.15	5.6±0.09	2.7±0.08 [2]
13 to 16 months	10	13.0±0.23	5.1±0.11	2.9±0.13
19 to 23 months	6	13.5±0.18	4.2±0.08	.. ...
All Ca analyses	83	12.6±0.18	.. ...	.. ...
All Mg analyses	68	... ...	.. ...	2.7±0.05

[1] Some animals were sampled more than once during this period.

[2] Magnesium analyses were made on only fourteen samples.

The calcium content of the serum of the foal at birth is likewise within the normal range for mature horses. Nevertheless there are greater changes in the calcium values than in those for magnesium. The calcium rises from 12.5 ± 0.19 mg. per 100 cc. of serum to 13.4 ± 0.21 mg. per 100 cc. at 12 weeks of age. Despite the fact that this difference, 0.9 mg., is statistically significant, both values are within the normal range which has been reported for mature horses. The mean value 13.5 ± 0.18 mg. per 100 cc. of serum for yearlings 19 to 23

months old is likewise within normal limits as reported by Pearson and Catchpole ('36).

The mean calcium value of 12.2 ± 0.15 mg. per 100 cc. of serum for the 6 to 12 months old foals is of interest. The samples were secured during the winter (December and February) of 1936 and (December) 1937. The figure given in the table represents the mean for samples taken on foals of both years. The low value is due entirely to the samples taken from the 1936 animals. The range for these samples was from 10.7 to 12.9 mg. with a mean of 11.9 ± 0.21 mg. The mean for the 1937 foals was 12.6 ± 0.45 mg. with a range from 12.0 to 13.6 mg. per 100 cc. of serum. The cause of this difference is not known. The mean 12.2 ± 0.15 for the entire group, while within the normal range, approaches some of the values reported by Millen and Eveleth ('38) for horses suffering from encephalomyelitis. The animals used in the present experiment, however, were normal and receiving their normal winter ration. This consisted of alfalfa hay and a grain mixture of oats, barley, and linseed meal. Except for the calcium analyses of the 1936 foals 6 to 12 months of age, all the other calcium analyses fall within the normal range for mature horses. The calcium content of the serum as well as the magnesium exhibits little change with increasing age.

At foaling time the inorganic phosphorus content of the blood serum was significantly higher for the foals than for the mares. The serum of the mares showed a mean inorganic phosphorus content of 3.3 ± 0.19 mg. per 100 cc. which is normal for mature horses. The value for the foals at birth, on the other hand, was 5.1 ± 0.15 mg. %. The analyses showed a rapid rise in the inorganic phosphorus content of the blood serum during the first 2 weeks after birth. This value reached a maximum of 7.9 ± 0.33 mg. % at 8 to 10 weeks of age. The amount of inorganic phosphorus present in the blood serum gradually decreased as the animals became older.

The table shows that the mean inorganic phosphorus values from 2 weeks to 1 year of age are higher than the mean value

for the foals at birth. Moreover, these differences are statistically significant. It is not until the animals are 13 to 16 months of age that the mean inorganic phosphorus value drops to the same level it was at birth. By the time the yearlings are 19 to 23 months old the amount of inorganic phosphorus present in the serum is significantly lower than it was at birth, although it is still higher than for the mares at foaling time.

The amount of inorganic phosphorus reported by Pearson ('34), 5.23 ± 0.13 mg. per 100 ml. serum, for animals under 1 year of age is similar to the amount, 5.6 ± 0.09 mg. found in the present data for foals 6 to 12 months old. This latter value is significantly higher than that for the amount of inorganic phosphorus present at birth. The present data in part corroborate Pearson's conclusion that a high negative correlation exists between age and inorganic phosphorus content of the blood serum. The reverse condition, however, is encountered when very young animals (birth to 10 weeks) are considered. In these animals it is seen that the inorganic phosphorus content of the serum increases as the animals approach 10 weeks of age.

SUMMARY

Calcium, inorganic phosphorus, and magnesium analyses of the blood serum of foals ranging from birth to 23 months of age were made. It was found that the amounts of calcium and magnesium present in the serum of these young animals were not different from those that have been reported for adult animals.

The inorganic phosphorus content of the foal's serum was higher at birth than that of the mare. There was a rapid rise during the first 2 weeks after birth in the amount of inorganic phosphorus found in the serum of the foal. A maximum was reached when the foals were about 10 weeks of age. From this point on the amount of inorganic phosphorus gradually decreased as the animals became older.

LITERATURE CITED

BRIGGS, A. P. 1924 Some applications of the colorimetric phosphate method. J. Biol. Chem., vol. 59, pp. 255–264.

CLARK, E. P., AND J. B. COLLIP 1925 A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., vol. 63, pp. 461–464.

DIMOCK, W. W., AND DANIEL J. HEALY 1933 Chemical character of the blood and urine of colts. J. Am. Vet. Med. Assoc., vol. 86, pp. 806–809.

ERRINGTON, B. J. 1937 Variations in the inorganic phosphorus and calcium content of the blood of horses. Cornell Vet., vol. 27, pp. 1–13.

EVELETH, D. F. 1937 Comparison of the distribution of magnesium in blood cells and plasma of animals. J. Biol. Chem., vol. 119, pp. 289–292.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

MILLEN, T. W., AND D. F. EVELETH 1938 Chemical changes in the serum associated with equine encephalomyelitis and moldy corn poisoning in horses. The Vet. Student, vol. 1, pp. 20–22.

PEARSON, P. B. 1934 Inorganic phosphorus in horse serum. J. Biol. Chem., vol. 106, pp. 1–6.

PEARSON, P. B., AND H. R. CATCHPOLE 1936 Correlated studies of the partition of calcium and inorganic phosphorus in the blood sera of Equidae. Am. J. Physiol., vol. 115, pp. 90–93.

CEREALS AND RICKETS

X. THE AVAILABILITY OF PHYTIC ACID PHOSPHORUS [1]

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A study of the interrelations of calcium, phosphorus, and vitamin D in the production of rickets has revealed that not all forms of phosphorus are equally available. For a time the total calcium and phosphorus relations were stressed, but of late the emphasis has shifted to comparisons of the availability of phosphorus in different forms. So far all the investigations bearing on the availability of phytic acid phosphorus, with the exception of that of Jones ('39), have failed to recognize the existence of various forms of phosphorus in cereal grain rations with the result that the data have been inconclusive or conflicting.

The object of the present experiments was to compare the availability of the phosphorus of phytic acid with phosphorus from inorganic sources. These were incorporated in a relatively simple ration, low in phosphorus, but complete in other respects. The resultant diets were fed to young rats and compared for their effect on bone ash, size of the costochondral junctions and the width of metaphyses.

EXPERIMENTAL

The basal ration used was the low phosphorus ration (R-14) of Schneider and Steenbock ('39). Its primary ingredients were cerelose 49.0, egg white 18, starch 20, Vitab 4, cottonseed

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

oil 5, low phosphorus salts 4%, respectively. This ration was supplemented with beta carotene fed in Wesson oil by dropper, 10 micrograms per rat per day. All other additions were made at the expense of the cerelose. The basal ration contained 0.04% phosphorus and 0.57% calcium as determined by analysis. The Ca content of the rations was kept constant at 0.57%, supplied as the carbonate (0.33%) and lactate (0.24%). The only variables in the ration were the kind and amount of phosphorus and the presence or absence of vitamin D.

The phytic acid was prepared from crude calcium phytate[3] according to the method of Boutwell ('17). It was added to the ration as a concentrated solution containing 133.8 mg. phosphorus per cubic centimeter. Of this 123 mg. was phytic acid phosphorus. Of the remainder, 6.34 mg. were shown to be inorganic phosphorus. The total and inorganic phosphorus were determined by the method of Fiske and Subbarow ('25) with an Evelyn photoelectric colorimeter. The phytic acid phosphorus was determined by the technique of McCance and Widdowson ('35). The phytic acid was found to be stable in water solution as no hydrolysis had occurred after 3 months standing although the color of its solution had deepened appreciably. The inorganic supplements were incorporated as an aqueous solution of equimolecular amounts of the mono- and dibasic sodium phosphates. The rations were dried before an electric fan at room temperature after addition of the supplements.

Nicolaysen's ('37) data were used in deciding the level of phosphorus to be fed. He pointed out that a 30 to 60 day old rat required 4.5 mg. phosphorus and 7.5 mg. calcium for each gram increase in body weight. Assuming an optimal rate of growth of 4 gm. per day on a food intake of 7 gm., 0.257 gm. "available" phosphorus and 0.428 gm. calcium were required per 100 gm. of ration. This was accepted as the optimal level of intake. Since some of the rats would not gain 4 gm. daily

[3] For this we are indebted to the E. A. Staley Manufacturing Co. of Decatur, Illinois.

even though they consumed 7 gm. of ration, suboptimal amounts of phosphorus were fed in addition.

In all, seven different rations were fed. These included, besides the optimal amount of phosphorus, levels of one-eighth, one-fourth, and one-half the optimal, plus a negative and positive control. The negative control group received as much inorganic phosphorus as was present as a contaminant in the largest addition of phytic acid. The positive control group received inorganic phosphorus equivalent to the total phosphorus. All the above rations were supplemented with 4.5 U.S.P. units of vitamin D (viosterol) per 100 gm. of ration mixed in ether solution. The ether was volatilized before an electric fan and the ration again mixed thoroughly. One additional ration with phytic acid containing one-fourth of the optimal level of phosphorus without vitamin D was included. Analyses of the rations after 2 weeks standing at room temperature revealed no hydrolysis of the phytic acid. The rations were kept in a refrigerator between feedings to avoid the development of rancidity.

Six 50 to 60 gm. rats were placed on each ration. Litter mates were distributed in the series so that no two rats of the same litter were on the same ration. They were kept individually in screen bottomed cages. They received distilled water ad libitum. A careful record of food consumption was kept; however, no attempts were made to equalize consumption. The rats were weighed and inspected weekly. At the conclusion of the experiment, the rats were anaesthetized and blood was drawn from the abdominal aorta for calcium and phosphorus determinations. The calcium was determined by the method of Clark and Collip ('25) and the phosphorus by the method of Fiske and Subbarow ('25). The radii and ulnae were removed for inspection of metaphyses, and the femora for a determination of ash after extraction with alcohol.

It is evident (table 1) that the inclusion of phytic acid in the low phosphorus ration caused a definite improvement in calcification. This became more evident as the amount of phytic acid phosphorus was increased to the optimal level. There

resulted a definite improvement in food consumption, in body weight, in weight of bone and bone ash, and in the percentage of bone ash. That this improvement was not due to the small amount of contaminating inorganic phosphorus is evident from the negative control (ration XII). The deficiency of phosphorus was also reflected in the blood picture. The average value for serum phosphorus for the negative control was

TABLE 1

The effect of increasing amounts of phytic acid phosphorus on calcification

RATION COMPOSITION (ADDITIONS TO BASAL)	NO. OF RATS	NO. OF WEEKS	FOOD CONS.	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	*gm.*	*gm.*	*gm.*	*%*
VII—0.032 gm.[1] (¼ opt.) phytic acid P plus D[2]	5	14	810	73	0.1773	0.0566	32.4
VIII—0.064 gm. (½ opt.) phytic acid P plus D	6	14	834	95	0.1770	0.0618	35.5
IX—0.128 gm. (½ opt.) phytic acid P plus D	6	14	1045	162	0.2594	0.1228	47.3
X—0.257 gm. (opt.) phytic acid P plus D	4	14	1146	186	0.3075	0.1620	56.3
X ———	2	10	715	135	0.4040	0.2275	52.8
XI—Same as VIII except no vitamin D	6	6	330	39	0.1140	0.0250	22.0
XII—0.0126 gm. inorganic P equal to that in X plus D	5	10	583	63	0.1323	0.0378	28.6
XIII—0.25 gm. (opt.) inorganic P plus D	4	14	1241	208	0.3993	0.2392	61.5
XIII ———	2	10	877	170	0.4615	0.2831	60.0

[1] Per 100 gm.

[2] 4.5 U.S.P. units vitamin D (viosterol) per 100 gm.

3.5 mg. % as compared with values ranging from 7 to 11 mg. % for the other groups. The serum calcium and phosphorus values for the other rats were of the order expected for mildly rachitic to normal rats.

The rats on the one-fourth optimal phosphorus level without vitamin D (ration XI) did so poorly that they were killed at the end of 6 weeks. Similarly the rats in the negative con-

trol group (ration XII) did poorly; they began to lose weight at the end of 10 weeks when they were killed. Since this latter group served as a control for the rats on the optimal phytic acid (ration X) and inorganic (ration XIII) phosphorus levels, representative rats from these two groups were also killed at the end of 10 weeks for comparative purposes. The remainder of the rats were continued for 14 weeks.

The striking difference between the rats on the one-fourth optimal phytic acid level without vitamin D (ration XI) and those receiving vitamin D (ration VIII) indicated the desirability of another experimental series. In this second series one-half optimal phytic acid and inorganic phosphorus with and without vitamin D were fed. As a negative control, inorganic phosphorus equivalent to that in the phytic acid ration was also fed. Four rats were placed on each ration under the same experimental conditions as in the first series except that the experiment was terminated at the end of 9 weeks. This modification was necessitated by the death of some of the rats during the seventh and eighth weeks. The data on the rats which died were not included in the tabulated results.

It is again evident (table 2) that vitamin D played an important role in the utilization of phytic acid phosphorus. Its effect on inorganic phosphorus was less pronounced. A comparison of the rats on one-half optimal inorganic phosphorus without D (ration XXIV) with those on the same ration plus D (ration XXIII) reveals that the former were only mildly rachitic and hence the increment of improvement which could possibly be achieved by the vitamin D was definitely decreased. A comparison of similar rations containing phytic acid phosphorus instead of inorganic phosphorus (rations IX and XXII) reveals that the rats which did not receive vitamin D were markedly rachitic. In fact the percentage of bone ash of the one-half optimal phytic acid phosphorus group without D (ration XXII) approximated the percentage of bone ash from the negative control rations (ration XXV and XXVI). Apparently under these conditions the phosphorus of phytic acid

was not demonstrably available. Vitamin D was without effect in the negative control group.

As no attempts were made to equalize the phosphorus intake of the rats in the different groups, the results suggested the desirability of carrying out a supplementary series of experiments in which this was done. In this the vitamin D additions were increased tenfold and the experiment was terminated at

TABLE 2

Effect of vitamin D on phytic acid and inorganic phosphorus utilization

RATION COMPOSITION (ADDITIONS TO BASAL)	NO. OF RATS	NO. OF WEEKS	FOOD CONS.	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	*gm.*	*gm.*	*gm.*	%
IX—0.128 gm.[1] (½ opt.) phytic acid P plus D[2]	4	9	556	113	0.2013	0.0916	45.4
XXII—Same as IX except no D	2	9	508	66	0.1572	0.0383	24.5
XXIII—0.128 gm. (½ opt.) inorganic P plus D	4	9	683	151	0.3338	0.1681	50.5
XXIV—Same as XXIII except no D	3	9	566	102	0.1440	0.0808	41.9
XXV—0.0063 gm. inorganic P equal to that in IX plus D	4	9	478	60	0.1237	0.0298	24.4
XXVI—Same as XXV except no D	2	9	441	15	0.0984	0.0231	23.5

[1] Per 100 gm.

[2] 4.5 U.S.P. units vitamin D (viosterol) per 100 gm.

the end of 4 weeks. It is evident from the results (table 3) that the presence of vitamin D had no effect on growth. However, vitamin D did have a marked effect on the utilization of phytic acid phosphorus (rations A and B) and a less marked effect on the utilization of inorganic phosphorus (rations C and D). These observations are in agreement with those of the second series. Although the food consumption was almost identical, the rats receiving the inorganic phosphorus, even without vitamin D, were definitely superior to those receiving the same amount of phosphorus as phytic acid with and without vitamin

D. Inspection of the metaphyses and costochondral junctions confirmed the above conclusions which in turn were in direct agreement with the percentage of bone ash. The calcium and phosphorus determinations on the blood sera failed to reveal any anomalies.

TABLE 3

Paired feeding study of the effect of vitamin D on phytic acid and inorganic phosphorus utilization

RATION COMPOSITION (ADDITIONS TO BASAL)	NO. OF RATS	NO. OF WEEKS	FOOD CONS.	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	*gm.*	*gm.*	*gm.*	%
A 0.128 gm.[1] (½ opt.) phytic acid P plus D[2]	6	4	215	39	0.1093	0.0405	37.0
C 0.128 gm. (½ opt.) inorganic P plus D	6	4	215	47	0.1482	0.0654	44.1
B Same as A except no D	6	4	211	39	0.0900	0.0194	21.5
D Same as C except no D	6	4	210	47	0.1280	0.0517	40.4

[1] Per 100 gm.

[2] 45 U.S.P. units vitamin D (viosterol) per 100 gm.

SUMMARY

When rats were fed a cereal free ration of normal calcium content the utilization of phosphorus from phytic acid was markedly enhanced by the addition of vitamin D. This effect was less marked with inorganic phosphorus. In no case was the utilization of phosphorus from phytic acid equal to that of inorganic phosphorus.

LITERATURE CITED

BOUTWELL, P. W. 1917 The phytic acid of the wheat kernel and some of its salts. J. Am. Chem. Soc., vol. 39, p. 491.

CLARK, E. P., AND J. B. COLLIP 1925 A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., vol. 63, p. 461.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

JONES, J. H. 1939 A comparison of cereal and non-cereal diets in the production of rickets. J. Nutrition, vol. 18, p. 507.

MCCANCE, R. A., AND E. M. WIDDOWSON 1935 CCCXX Phytin in human nutrition. Biochem. J., vol. 29, p. 2694.

NICOLAYSEN, R. 1937 XIII A note on the calcium and phosphorus requirement of rachitic rats. Biochem. J., vol. 31, p. 105.

SCHNEIDER, H. A., AND H. STEENBOCK 1939 A low phosphorus diet and the response of rats to vitamin D_3. J. Biol. Chem., vol. 128, p. 159.

CEREALS AND RICKETS

XI. CALCIUM PHYTATE AS A SOURCE OF CALCIUM[1]

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It is generally assumed that the calcium of compounds ordinarily used to supplement grain rations is entirely available. Steenbock et al. ('23) in their experiments with young rats found the calcium of calcium lactate, carbonate, phosphate, silicate, and sulfate to be available to the rat. Buckner and Martin ('29) observed that calcium carbonate, phosphate, and sulfate served equally well as sources of calcium for the growing chick. Bethke, Kennard and Kick ('29) confirmed the work of Buckner and Martin. They extended their studies to the calcium of limestone, steamed bone meal, rock phosphate, phosphatic limestone, and oyster shells, and found no differences in assimilation. However, Mellanby ('37) is of the opinion that phytic acid actually increases rickets in dogs on moderately rachitogenic diets by the withdrawal of calcium from the body tissues. There is the possibility that even the calcium of calcium phytate taken by mouth might be unavailable. This possibility was accordingly put to test. Mellanby's theory regarding the withdrawal of calcium from the tissues by unhydrolyzed phytic acid will be given consideration in a subsequent paper.

EXPERIMENTAL

The calcium phytate used was prepared from crude calcium phytate[2] according to the method outlined by Anderson ('12).

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] Received from E. A. Staley Manufacturing Co. of Decatur, Ill.

Analyses revealed it to contain 16.9% calcium and 17.0% phosphorus. Of the phosphorus, 0.54% was present in the inorganic form. The basal ration in which it was incorporated was essentially the purified low phosphorus ration of Schneider and Steenbock ('39) with the addition of optimal amounts of mono- and di-potassium phosphates, except for the omission of the calcium salts. The composition of the modified salt mixture was as follows: NaCl 183.7, K_2HPO_4 187.83, KH_2PO_4 147.02, KCl 17.3, $MgSO_4$ 90.0, $MnSO_4$ 0.20, $K_2Al_2(SO_4)_4 \cdot 24H_2O$ 0.09, $CuSO_4 \cdot 5H_2O$ 0.39, NaF 0.57, KI 0.05 and Fe citrate 16.15 parts, by weight, respectively.

Two and fifty-seven hundreds grams were incorporated in each 100 gm. of ration. The basal ration contained 0.019% calcium as determined by the method of Meloche, Clifcorn, and Griem ('33).

The level of calcium to be fed was arrived at on the assumption that the calcium was entirely available (Krieger, Bunkfeldt and Steenbock, '40). Calcium as the carbonate or phytate, to the extent of 0.428 gm., was incorporated in each 100 gm. of ration for the optimal level. The experimental conditions and criteria were the same as those of the previous investigation.

A preliminary experiment of 13 weeks' duration in which calcium phytate was fed at the optimal level and at levels of one-half, one-fourth, and one-eighth of t. e optimal, revealed that in the presence of vitamin D there was a marked improvement in growth, food consumption, weight of bone, weight of bone ash, and consequently, percentage of bone ash with an increase in the calcium intake up to one-half the optimal level. Little or no difference was observed between the rats in the positive control group receiving calcium carbonate and those receiving the same amount of calcium as calcium phytate. However, when vitamin D was omitted from the calcium phytate ration, calcification was markedly reduced. The percentage of bone ash for the group on one-fourth the optimal calcium intake without vitamin D was 45.3% as compared with 54.8% for the same ration supplemented with vitamin D. As calcium car-

bonate was not fed at an identical level without vitamin D, it appeared desirable to run a second experimental series in which this was done. The experimental conditions in this series were essentially the same as in the first.

It is evident (table 1) that in this series the calcium of calcium phytate was utilized as readily as that of calcium carbonate regardless of whether vitamin D was present or not.

TABLE 1

Comparative study of the availability of calcium in calcium phytate and calcium carbonate

	ADDITIONS TO THE BASAL RATION [1]	FOOD CONS.	CHANGE IN WT.	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
		grams	*grams*	*grams*	*grams*	%
XIV	0.107 gm. (1/4 opt.) Ca as Ca phytate plus D[2]	728	130	0.2588	0.1314	50.8
XVI	0.107 gm. (1/4 opt.) Ca as $CaCO_3$ plus D	692	127	0.2630	0.1290	49.1
XV	Same as ration XIV except no vitamin D	535	76	0.2005	0.0856	42.5
XVII	Same as ration XVI except no vitamin D	548	58	0.1588	0.0691	43.5
XVIII	0.054 gm. (1/8 opt.) Ca as Ca phytate plus D	663	102	0.2102	0.0878	40.9
XX	0.054 gm. (1/8 opt.) Ca as $CaCO_3$ plus D	582	76	0.1776	0.0730	41.5
XIX	Same as ration XVIII except no vitamin D	502	46	0.1576	0.0579	36.8
XXI	Same as ration XX except no vitamin D	493	53	0.1582	0.0582	35.9

[1] Each ration was fed to six rats for 12 weeks. The additions represent those made for 100 gm. of ration.

[2] 4.5 U.S.P. units vitamin D (viosterol) per 100 gm.

Vitamin D produced a definite increase in growth, food consumption, weight of bone and weight of bone ash and consequently percentage of bone ash at the one-eighth and one-fourth optimal levels. This improvement was probably due to the increased absorption of calcium from the gut as has been demonstrated by Nicolaysen ('37). It was again obvious that the amount of calcium present was the limiting factor

since the higher levels (rations XIV, XV, XVI, and XVII) produced the best growth and calcification. This was also borne out by the blood picture. The serum phosphorus values were normal in all cases, but the calcium values were 3 to 5 mg.% below those obtained at the one-half optimal and optimal levels in the preliminary experiment. The metaphyses and the costochondral junctions revealed normal or almost normal calcification except in those animals which received one-eighth the optimal levels without vitamin D.

SUMMARY

When calcium phytate was added to a cereal-free low-calcium ration, its calcium was found to be as readily available to the rat as the calcium of calcium carbonate. Vitamin D improved the utilization of both forms of calcium to the same extent.

LITERATURE CITED

ANDERSON, R. J. 1912 Phytin and phosphoric acid esters of inosite. J. Biol. Chem., vol. 12, p. 97.

BETHKE, R. M., D. C. KENNARD AND C. H. KICK 1929 The availability of calcium in calcium salts and minerals for bone formation in the growing chick. Poultry Sci., vol. 9, p. 45.

BUCKNER, G. D., AND J. H. MARTIN 1929 Calcium and phosphorus metabolism of the growing chick. Poultry Sci., vol. 8, p. 284.

KRIEGER, C. H., R. BUNKFELDT AND H. STEENBOCK 1940 Cereals and rickets. X. Availability of phytic acid phosphorus. J. Nutrition, vol. 20, p. 7.

MELLANBY, E. 1937 Toxamins in foods. Perspectives of Biochemistry, Cambridge University Press, p. 318.

MELOCHE, V. W., L. E. CLIFCORN AND W. B. GRIEM 1933 Determination of calcium in mineral mixtures. J. Ass. Off. Agri. Chem., vol. 16, p. 240.

NICOLAYSEN, R. 1937 Studies upon the mode of action of vitamin D. III. The influence of vitamin D on the absorption of calcium and phosphorus in the rat. Biochem. J., vol. 31, p. 122.

SCHNEIDER, H. A., AND H. STEENBOCK 1939 A low phosphorus diet and the response of rats to vitamin D_2. J. Biol. Chem., vol. 128, p. 159.

STEENBOCK, H., E. B. HART, M. T. SELL AND J. H. JONES 1923 The availability of calcium salts. J. Biol. Chem., vol. 56, p. 375.

CALCIUM AND PHOSPHORUS METABOLISM IN RATS AND DOGS AS INFLUENCED BY THE INGESTION OF MINERAL OIL

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Although conclusive data showing the interference of mineral oil with the utilization of vitamin A, both as such and in its precursor form, carotene, have been offered (Burrows and Farr, '27; Dutcher et al., '34; Jorstad and Johnston, '26; Mitchell, '33; Rowntree, '31; Anderson, '39 and Curtis and Kline, '39), the evidence on the effect of mineral oil ingestion upon the availability of vitamn D is conflicting. Much of this evidence has resulted from a search for the best diluent for vitamin D concentrates for use as an antirachitic agent (Dutcher et al., '27; Hawks et al., '29; Jackson, '31, '34 a, 34 b).

The experiments presented in this paper were designed to gain further information regarding the influence of mineral oil ingestion by rats and also by dogs upon the metabolism of calcium and phosphorus in these species. In rats, the effect of continuous ingestion of mineral oil upon calcium and phosphorus metabolism has been studied by comparing the degree of calcification of rachitic lesions (as shown by the "line test") afforded by various levels of vitamin D fed as cod liver oil in the presence and absence of mineral oil. In dogs, continuous calcium and phosphorus "balance studies" were conducted for 11 weeks.

EXPERIMENTAL

Amount and method of administration of mineral oil

In order that the results of this work might have practical significance, consideration of the therapeutic dose for humans was made in the selection of the amount of mineral oil to be fed to the experimental animals. The difficulty, however, of arriving at a comparable therapeutic dose for rats is apparent when the differences in size, food intake, eating habits and rate of growth and development are realized.

In the experiments described herein, the mineral oil [1] was incorporated in the basal ration at two different percentage levels, i.e., 5 and 10%. In this way, the amount of mineral oil ingested was automatically proportional to the food or calorie intake (Jackson, '31), and lubrication was continuous. The fat soluble vitamin supplements were fed separately (Jackson, '34 a). Food consumption records showed an average daily intake of mineral oil by the rats of from 0.2 to 0.5 cc.

Jackson ('31) and Rowntree ('31) have independently estimated that 0.5 cc. of mineral oil daily per rat was comparable to an average human therapeutic dose of 30 cc. This amount of mineral oil was found to cause no diarrhea. The feces were bulkier and softer but preliminary tests using carmine as a marker, showed that even the higher level of mineral oil ingestion produced slight, if any, acceleration in the rate of elimination (Smith and Spector, '40).

EXPERIMENTS WITH RATS

Young rats weighing from 55 to 65 gm. were placed upon Steenbock's high-calcium low-phosphorus rickets-producing ration no. 2965 which is composed of ground yellow corn 76, wheat gluten 20, calcium carbonate 3, and sodium chloride 1%, respectively. When care was taken to keep the rats in a dark room from which all direct light was excluded, the animals regularly developed severe rickets in 21 days as indicated by

[1] The mineral oil used was a heavy California liquid petrolatum product of E. R. Squibb and Sons.

the findings of the "line test" developed by McCollum and his co-workers ('22). At this time, two or more representative rats from each litter were continued as controls upon the rickets-producing ration no. 2965 whereas the remainder of the litter were given the same ration in which mineral oil was incorporated. Each rat was housed individually in a round metal cage with a raised screen bottom and given the basal diet and distilled water ad libitum.

In addition to the basal ration, each rat received a daily feeding of cod liver oil which was introduced by pipette directly into its mouth. No difference in the results obtained was found when the vitamin D supplement was given at 8 A.M. or 6 P.M. after a day of fast. The cod liver oil was so diluted with corn oil that 0.05 cc. fed daily during a 10-day test period produced a continuous line of calcification at the zone of provisional calcification in the rachitic metaphyses of the control animals. Using the Bills et al. ('31) system for grading the degree of healing of the rachitic lesions, it may be seen in table 1 that an average of 2.4+ healing was recorded for the thirty-six rats receiving 0.05 cc. of the cod liver oil solution.

Graded amounts of this same oil dilution were given to the litter mate mineral oil-fed rats and the degree of calcification of the rachitic lesions after a 10-day feeding period was again judged according to the same standards.

The data which are summarized in table 1 plainly show that an amount of cod liver oil, which produced a continuous line of calcification in the rachitic metaphyses of the rats receiving the basal diet 2965 alone, produced no healing whatsoever when mineral oil incorporated in the same ration was ingested. When the amount of cod liver oil fed was increased, some healing occurred, but it may be seen that at least three times as much vitamin D as cod liver oil was necessary to produce the same degree of calcification when 5% mineral oil was present in the ration. Again, between five and ten times as much cod liver oil was necessary to compensate for the ingestion of mineral oil at the higher level. Thus, proof

was afforded of an interference with the utilization of the vitamin D in cod liver oil, and therefore of a decrease in the antirachitic potency of cod liver oil resulting from the ingestion of mineral oil. Records of the food consumption of these rats showed an average food intake of slightly less than 50 gm. during the test period. It is evident, therefore, that the amount of mineral oil ingested even at the higher level of incorporation in the ration was not excessive and was probably less than a comparable therapeutic dose for humans.

TABLE 1

Summarized "line test" findings showing the effect of mineral oil ingestion upon the calcification of rachitic lesions induced by cod liver oil

AMOUNT OF COD LIVER OIL FED	DIET 2965 ALONE			DIET 2965 PLUS 5% MINERAL OIL			DIET 2965 PLUS 10% MINERAL OIL		
	Number of rats	Average food intake	Average degree of healing [1]	Number of rats	Average food intake	Average degree of healing [1]	Number of rats	Average food intake	Average degree of healing [1]
cc.		*gm.*			*gm.*			*gm.*	
0.05	36	48	24+	11	46	0.0	12	47	0.0
0.1				12	48	1.8+	7	43	0.0
0.10				9	46	2.4+	13	45	1.6+
0.25				4	49	2.8+	13	47	2.0+
0.5				3	48	4.0+	3	48	2.8+

[1] Bills et al. ('31).

EXPERIMENT WITH DOGS

Although most workers have used rats for the study of factors affecting the production of rickets and especially for vitamin D assays, Mellanby ('19) has used dogs for the study of experimental rickets, believing that the course of rickets in puppies more closely resembles that in human beings. In dogs, as in children, a deficiency of vitamin D alone is sufficient to produce rickets without any distortion of the diet in other respects, whereas in rats, the calcium and phosphorus relationship in the diet must be abnormal as well.

Accordingly, a study has been made of the effect of mineral oil ingestion by puppies receiving a ration probably optimum in calcium and phosphorus content and adequately provided with vitamin D. In this phase of the investigation, the effect

of mineral oil ingestion has been observed by means of continuous calcium and phosphorus "balance experiments" for an 11-week period during the ages of very rapid growth and development.

Methods and materials

Four mongrel puppies (Scottie mother) weaned at 4 weeks of age were given 47.5 gm. per kilo of a ration similar to Morgan and Garrison's ('30) modification of the Karr-Cowgill laboratory ration for dogs. The constituents of this ration were thoroughly mixed and then thinned with distilled water to a mushy consistency at the time of feeding. The diet was supplemented by the daily feeding of 400 U.S.P. units of vitamin A [2] per kilo. Previous findings of this laboratory (Bradfield and Smith, '38) showed that normal growth of puppies was provided for, and a slight storage of vitamin A in the liver was permitted, when half this amount of vitamin A was administered daily. At this level of ingestion, carotene as a source of vitamin A appeared to be equally as well utilized as cod liver oil.

Vitamin D was fed separately as cod liver oil to all the dogs, except the negative control animal no. 35, in the amount of 1 U.S.P. unit per kilo of body weight per day (Arnold and Elvehjem, '37).

When the puppies had become accustomed to their basal ration and were observed to be developing normally, they were housed in individual wire mesh metabolism cages designed to permit collection of both the urine and feces. The cages were protected against strong sunlight by enclosing them in heavy brown paper and keeping them in the laboratory after the experimental regime was begun.

Dog 35 was placed on the vitamin A supplemented basal ration without vitamin D, thus serving as the negative control. Dog 36 was given the basal ration with both vitamin A and D supplements fed separately and served as the positive control. Dog 37 was given the basal ration in which 5%

[2] Given as S M A C O, a commercial solution of carotene in oil.

mineral oil was incorporated, and received vitamins A and D fed separately. Dog 38 subsisted on the basal ration in which 10% mineral oil was incorporated, with vitamin A and D supplements fed separately in the same amounts as given to the other dogs.

All of the puppies received distilled water ad libitum. They were fed once a day in the morning and the vitamin supplements were given in the late afternoon. In most cases the food was quickly consumed but in instances in which part of it was refused after allowing a reasonable period for its consumption, it was returned to the refrigerator and fed the next day before a fresh allotment was given. A record of the food consumed by each dog was kept for the duration of the experimental period.

The feces and urine were collected daily, and at the end of each 7-day balance period each cage was washed with distilled water and the wash water added to the total collection for the week. Aliquots of the homogenous mixture were analyzed for calcium by the method of McCrudden ('12) and for phosphorus by the method of Fiske and Subbarow ('25).

Samples of the basal ration were analyzed by the same methods and these analyses in combination with the food consumption records gave the basis for data concerning the calcium and phosphorus intakes of the dogs during each 7-day balance period.

Results

Summarized results of the calcium and phosphorus balance studies in which dogs were used are presented in table 2.

It may be seen that in every balance period, the mineral oil fed dogs retained less calcium and phosphorus than did the control animal which received no mineral oil, whether the results are expressed in terms of the absolute amount of these elements retained, or the amount retained per kilo of body weight, or the percentage of the ingested calcium and phosphorus which was retained in the body. In most balance

TABLE 2

The effect of mineral oil ingestion upon the metabolism of calcium and phosphorus in dogs

SERIES AND PERIOD	DOG NO.		CALCIUM RETENTION			PHOSPHORUS RETENTION		
			Total per day	Per cent of intake	Per kilogram per day	Total per day	Per cent of intake	Per kilogram per day
			gm.		*gm.*	*gm.*		*gm.*
A-1[1] Age 65–71 days	35	Negative control	0.85	23.8	0.0224	0.49	11.3	0.0108
	36	Positive control	1.94	71.1	0.1379	1.17	35.0	0.0422
	37	5% mineral oil	1.16	61.2	0.0396	0.60	24.5	0.0204
	38	10% mineral oil	2.73	52.2	0.0674	2.00	31.5	0.0495
A-2 Age 72–75 days	35	Negative control	4.10	84.1	0.1379	2.91	48.9	0.0977
	36	Positive control	2.95	69.0	0.1094	4.14	79.3	0.1534
	37	5% mineral oil	2.18	61.8	0.0736	2.06	47.9	0.0695
	38	10% mineral oil	4.50	59.7	0.1003	4.12	44.8	0.0919
A-3 Age 79–85 days	35	Negative control	5.63	88.5	0.1550	5.94	75.5	0.1635
	36	Positive control	3.16	52.5	0.0622	2.71	36.8	0.0533
	37	5% mineral oil	5.64	85.9	0.1365	2.41	30.1	0.0583
	38	10% mineral oil	1.95	50.3	0.0524	1.23	26.1	0.0301
A-4 Age 86–92 days	35	Negative control	1.76	44.3	0.0492	1.26	26.0	0.0353
	36	Positive control	4.63	55.7	0.0682	4.02	39.7	0.0750
	37	5% mineral oil	6.77	85.1	0.1470	6.64	68.4	0.1443
	38	10% mineral oil	1.33	57.4	0.0372	0.63	22.3	0.0176
A-5 Age 93–99 days	35	Negative control	1.98	51.7	0.0525	1.71	36.7	0.0454
	36	Positive control	3.71	47.4	0.0588	2.21	23.1	0.0350
	37	5% mineral oil	6.61	90.4	0.1215	4.95	55.6	0.0919
	38	10% mineral oil	1.01	44.0	0.0268	0.28	10.2	0.0075
A-6 Age 100–106 days	35	Negative control	1.09	38.0	0.0270	0.80	22.8	0.0197
	36	Positive control						
	37	5% mineral oil						
	38	10% mineral oil						

TABLE 2—*Continued*

SERIES AND PERIOD	DOG NO.	CALCIUM RETENTION			PHOSPHORUS RETENTION		
		Total per day	Per cent of intake	Per kilogram per day	Total per day	Per cent of intake	Per kilogram per day
		gm.		*gm.*	*gm.*		*gm.*
B-1[2] Age 107–113 days	35 Negative control	6.11	65.2	0.0953	4.85	42.5	0.0968
	36 Positive control	7.49	95.9	0.1309	6.30	66.2	0.1102
	37 5% mineral oil	3.61	71.2	0.0948	3.36	54.4	0.0882
	38 10% mineral oil	1.87	53.1	0.0461	1.95	45.4	0.0482
B-2 Age 114–120 days	35 Negative control	8.67	94.4	0.1221	6.72	60.0	0.0924
	36 Positive control	7.54	93.9	0.1144	5.75	58.7	0.0874
	37 5% mineral oil	5.58	79.4	0.1244	4.78	55.7	0.1065
	38 10% mineral oil	3.65	69.3	0.0821	3.14	48.9	0.0707
B-3 Age 121–127 days	35 Negative control	8.62	95.2	0.1180	5.96	53.9	0.0817
	36 Positive control	8.34	95.4	0.1251	6.03	56.5	0.0904
	37 5% mineral oil	7.77	94.9	0.1491	5.71	57.1	0.1161
	38 10% mineral oil	5.42	71.9	0.1171	4.86	53.0	0.1053
B-4 Age 128–134 days	35 Negative control	8.42	93.2	0.1058	7.81	70.8	0.0984
	36 Positive control	7.93	95.1	0.1056	7.11	69.9	0.0946
	37 5% mineral oil	6.62	79.9	0.1150	5.96	59.0	0.1035
	38 10% mineral oil	....	...	.....	....	...	.....
B-5 Age 135–141 days	35 Negative control	10.71	95.0	0.1252	10.80	78.6	0.1263
	36 Positive control	9.03	94.5	0.1146	8.90	76.3	0.1129
	37 5% mineral oil	5.88	73.0	0.0949	6.30	64.2	0.1018
	38 10% mineral oil	3.81	51.8	0.0671	5.15	57.4	0.0908

[1] In balance series A, all dogs were fed 1 U.S.P. unit of vitamin D per kilogram per day except negative control no. 35.

[2] In balance series B, all dogs were fed 5 U.S.P. units of vitamin D per day including the negative control no. 35.

periods the dogs receiving the mineral oil retained even less calcium and phosphorus than did the negative control dog which was given no cod liver oil supplement of vitamin D. For example, in the first balance study of 6 weeks' duration, during which time the dogs received 1 U.S.P. unit of vitamin D per kilo of body weight daily, the mineral oil fed puppies retained only about a third as much calcium and phosphorus as did the positive control dog given the same basal ration and the same amount of the vitamin D supplement. Expressed in relation to body weight, the positive control dog each day retained an average of 0.0181 gm. of calcium and 0.0142 gm. of phosphorus per kilo as compared with an average daily retention of 0.0069 gm. of calcium and 0.0054 gm. of phosphorus by the litter mate dog whose ration contained 10% mineral oil. Thus, in spite of supposedly adequate intakes of both calcium and phosphorus and of the vitamin which regulates the metabolism of these mineral elements, the mineral oil fed dogs were not able to make satisfactory use of these essential dietary constituents probably because of the interference with the utilization of vitamin D as a result of the mineral oil ingestion.

This inability was also clearly reflected in the general nutritive condition of the puppies. The characteristic clinical evidences of severe rickets were apparent in the negative control dog which was given no vitamin D, and even more severe symptoms were noted in the mineral oil fed puppies which received 1 U.S.P. unit of vitamin D per kilo of body weight daily in the form of cod liver oil. These puppies showed the flat footed condition, bowing of the forelegs, rachitic posture of the hind legs, swelling of the wrist and ankle joints, general weakness and inability to support the weight of the body, which are the characteristics of severe rickets. In addition they became very timid, displayed signs of pain and developed thin straggly hair, the latter characteristic also of vitamin A deficiency. The negative control dog was decidedly bow-legged but was more active and had a thicker, better appearing coat of fur than did its mineral oil fed litter mates.

In the second series of balance studies on the same dogs, the level of feeding of vitamin D was increased fivefold, i.e., 5 U.S.P. units per kilo were given daily. In order to have a basis for comparison of the curative effect of this increased dosage in the presence and absence of mineral oil in the ration, the negative control rachitic dog no. 35 was given the same relatively high dose of cod liver oil as the mineral oil fed dogs and their comparative metabolism of calcium and phosphorus observed as before. The results are summarized as balance series B in table 2.

When vitamin D as cod liver oil was given to dog 35 which previously served as the negative control, the retention of both calcium and phosphorus immediately increased. The average daily retention of calcium increased from 0.0094 gm. per kilo in the first period when no cod liver oil was given to 0.0162 gm. in the second period. The percentage of ingested calcium which was retained increased from 45 to 88%, and there were accompanying parallel increases in the retention of phosphorus. Dog 35 was retaining therefore, practically as much of these mineral elements as dog 36 which had always served as the positive control. These changes were reflected in the outward characteristics of dog 35 which changed from an inactive, unsteady, timid animal with severely bowed forelegs to a lively dog which appeared normal except for the enlarged wrist and ankle joints, evidences of his previous rachitic condition. Thus, ample evidence was afforded that the basal ration was rendered complete by the addition of vitamin D as cod liver oil.

Again it may be noted, however, that the retentions of calcium and phosphorus by the mineral oil fed dogs were not optimal. This was especially true in the case of dog no. 38 which received the basal ration containing 10% mineral oil. Increasing fivefold the amount of cod liver oil fed to this dog, although resulting in a greatly increased retention of the bone forming minerals, and a decided improvement in the nutritive condition of the animal, did not lead to optimal retention. Dog 38 in the second period retained somewhat less than two-thirds the amount of calcium and phosphorus

retained by the rachitic negative control dog which was given the same dosage of vitamin D. Here again, as in the case of the rachitic rats previously discussed, the apparent potency of cod liver oil in the healing of rachitic lesions was diminished by the presence of mineral oil in the alimentary canal.

SUMMARY AND CONCLUSIONS

The effect of mineral oil ingestion on the metabolism of calcium and phosphorus has been studied in rats by comparing the ability of vitamin D fed as cod liver oil, to calcify rachitic lesions in the presence and absence of mineral oil, and in dogs, by comparing the retention of calcium and phosphorus by means of a series of weekly balance studies in mineral oil and non-mineral oil fed puppies. The following observations were made:

1. Mineral oil ingestion interfered with the utilization of vitamin D fed separately as cod liver oil to such an extent that three times as much cod liver oil was necessary to induce healing of the rachitic lesions of rats when the basal ration contained 5% mineral oil, and somewhere between five and ten times as much was needed when 10% mineral oil was incorporated in the basal ration.

2. The balance studies showed that mineral oil ingestion by young dogs interfered with the retention of both calcium and phosphorus so seriously that normal calcification of the bony structure was not possible, the mineral oil fed dogs showing the characteristics of severe rickets even though they received adequate amounts of calcium and phosphorus and were given a supposedly minimum protective dose of cod liver oil. Increasing the amount of cod liver oil fivefold did not provide for optimal retention of the mineral elements in the dog receiving the ration containing 10% mineral oil.

LITERATURE CITED

ANDERSON, OLAF 1939 The effect of paraffin oil on resorption of vitamin A in man. Klin. Wochschr., vol. 18, pp. 499–502.

ARNOLD, A., AND C. A. ELVEHJEM 1937 Studies on nutritive efficiency of commercial dog foods. J. Am. Vet. Med. Assoc., vol. 44, pp. 515–531.

BILLS, C. E., E. M. HONEYWELL, A. M. WIRECK AND M. NUSSMEIER 1931 A critique of the "line test" for vitamin D. J. Biol. Chem., vol. 85, pp. 687–711.

BRADFIELD, D., AND M. C. SMITH 1938 The ability of the dog to utilize vitamin A from plant and animal sources. Am. J. Physiol., vol. 124, p. 168.

BURROWS, M. T., AND W. K. FARR 1927 The action of mineral oil per os on the organism. Proc. Soc. Exp. Biol. and Med., vol. 24, pp. 719–723.

CURTIS, A. C., AND ED. M. KLINE 1939 Influence of liquid petrolatum on the blood content of carotene in human beings. Archives Int. Med., vol. 63, pp. 54–63.

DUTCHER, A. R., J. O. ELY AND H. E. HONEYWELL 1927 Assimilation of vitamin A and D in the presence of mineral oil. Proc. Soc. Exp. Biol. and Med., vol. 24, pp. 953–955.

DUTCHER, A. R., P. L. HARRIS, E. R. HARTZLER AND B. GUERRANT 1934 The assimilation of carotene and vitamin A on the presence of mineral oil. J. Nutrition, vol. 8, pp. 269–283.

FISKE, C. H., AND W. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, pp. 375–400.

HAWKS, P. B., H. LEVINE, C. J. STUCKY AND B. L. OSER 1929 The effect of mineral oil upon the utilization of vitamin A and D in cod liver oil. Abstract of paper. Am. Chem. Soc., Minneapolis.

JACKSON, RICHARD W. 1931 The effect of mineral oil administration upon the nutritional economy of the fat soluble vitamins. I. Studies with the vitamin A of butterfat. J. Nutrition, vol. 4, pp. 171–184.

———— 1934 a II. Studies with the vitamin A factor of yellow corn. J. Nutrition, vol. 7, pp. 607–616.

———— 1934 b III. Studies with vitamin D of irradiated ergosterol. J. Nutrition, vol. 7, pp. 617–622.

JORSTAD, L. D., AND C. G. JOHNSTON 1926 Reaction produced by lipoid solvents in animals fed diets deficient and high in vitamin A. Proc. Soc. Exp. Biol. and Med., vol. 24, pp. 86–88.

MCCOLLUM, E. V., N. SIMMONDS, P. C. SHIPLER AND E. A. PARK 1922 A delicate biological test for calcium depositing substances. J. Biol. Chem., vol. 51, p. 41.

MCCRUDDEN, F. D. 1912 The determination of calcium in the presence of magnesium and phosphates. J. Biol. Chem., vol. 10, pp. 187–199.

MELLANBY, E. 1919 An experimental investigation of rickets. Lancet, vol. 1, p. 407.

MITCHELL, HELEN S. 1933 The influence of mineral oil on assimilation of vitamin A from spinach. Proc. Soc. Exp. Biol. and Med., vol. 31, p. 233.

MORGAN, A. F., AND A. E. GARRISON 1930 The effect of vitamin D and of reaction of diet upon response to parathyroid extract. J. Biol. Chem., vol. 85, pp. 687–711.

ROWNTREE, JENNIE I. 1931 The effect of the use of mineral oil upon the absorption of vitamin A. J. Nutrition, vol. 3, pp. 345–351.

SMITH, M. C., AND H. SPECTOR 1940 Some effects on animal nutrition of the ingestion of mineral oil. Arizona Agricultural Experiment Station Bull. 84.

TASTE THRESHOLDS AND TASTE PREFERENCES OF RATS FOR FIVE COMMON SUGARS [1]

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FIVE FIGURES

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In previous experiments taste thresholds for salt and sucrose were determined for rats and humans. Taste thresholds have now been determined for other sugars—maltose, glucose, galactose, and lactose. In addition, a study was made of the taste preferences for these five sugars. The present study deals with the results obtained with rats; the results of a similar study made on humans will be reported later.

Before presenting these results a brief account of our previous experiments will help to orient our interest in taste thresholds. Until recently taste studies were purely of academic interest with little or no relationship to problems of nutrition. The results of our observations have shown that taste may play an important part as a guide to nutritional needs.

Our studies on self-selection of diets are based on the concept of the internal environment first advanced by Claude Bernard (1859). Bernard stated then that, "It is the fixity of the 'milieu interne' which is the condition of free and independent life; and all the vital mechanisms, however varied they may be, have only one object, that of preserving

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constant the condition of life in the internal environment." Bernard, and later Cannon, who greatly extended this concept ('32), concerned themselves with the physiological mechanisms which stabilize the internal environment, such as, for instance, changes in pulse rate, blood pressure, fat metabolism, etc.

In our studies we have found that behavior factors, or responses of the total organism, may also contribute to this end. One example will suffice to illustrate what is meant by behavior factors. In the normal organism the regulation of the sodium balance depends on the secretions from the cortex of the adrenal glands (Loeb, '33; Harrop et al., '33). After removal of these glands, the body excretes large amounts of sodium in the form of the chloride salt, and the internal environment becomes changed to such an extent that the organism cannot survive; the rats develop symptoms of insufficiency and die in 10 to 20 days. Administration of sodium chloride, either by injection or by feeding, will keep the adrenalectomized rats alive. We were interested to know whether, if given free access to salt in a container separate from the food, adrenalectomized rats would of their own accord take a sufficient amount of salt to maintain a normal internal salt balance and thus keep themselves alive. For this purpose rats were kept in cages which contained a food cup and two graduated bottles, one filled with a 3% solution of sodium chloride, the other with tap water. Records were made daily of the fluid intake. After base lines were obtained of the fluid intake, we removed the adrenals. Very shortly after operation, in some instances even on the same day, the rats began to drink more salt solution (Richter, '36). After 30 to 40 days their daily intake averaged five to twenty times above their preoperative level. The rats survived and gained weight. Thus, after the internal or physiological means of regulation of the sodium balance had ceased to function, the animals themselves took care of the balance by ingesting large amounts of salt.

We do not know the relative importance of the roles played by the physiological and behavior factors in the normal organism. The experiments in this laboratory have shown that these behavior factors participate in a wide range of functions, regulation of calcium metabolism after parathyroidectomy (Richter and Eckert, '37), fat and carbohydrate metabolism after pancreatectomy (Richter and Schmidt, '40), and similar metabolic disturbances in vitamin B deficiency (Richter and Barelare, '39). The rats show normal growth, reproduction, and activity on diets which they select from purified substances—minerals, crystallized vitamins, purified fats, carbohydrates, and proteins (Richter, Holt and Barelare, '38).

Having demonstrated that rats make beneficial dietary selections from purified substances, little doubt remained that natural foods must also be selected by them on the basis of taste. With natural foods the situation is more complicated because of the many different ingredients—minerals, vitamins, etc. When an animal or a human craves a certain food, we do not know which ingredient or ingredients he wants. We could narrow down the field if we knew which of the ingredients were present in a sufficiently large amount to be tasted, that is, in concentrations above the taste threshold.

To supply this knowledge the taste thresholds of rats and humans for many different substances, both beneficial and harmful, have been determined. These studies have opened up an entirely new approach to the problem of the relation of the taste of substances to their nutritional value.

The technique which was used for the experiments on rats is quite simple. The cages were of the same type as in the adrenalectomy and salt appetite experiments. Each cage contained two graduated inverted bottles. In these experiments distilled water was put in both bottles for the first 10 to 15 days. At the end of this time the daily intake from each bottle had usually reached a fairly constant amount. Then one bottle was filled with a very low concentration of the solution to be tested, and thereafter the strength of the solution was increased daily in small steps. We have found so

far that with substances which are needed for normal nutrition, such as sodium chloride, sodium phosphate, potassium chloride, and sucrose, when the concentrations reach a certain level, the rats will begin drinking more of the solution and less of the water, while with harmful or poisonous solutions, such as those of mercuric chloride and arsenic trioxide, when the concentrations reach a certain point, the rats take less of the solution and more of the water. The point at which the rats first indicated that they recognized a difference between the distilled water and the solutions was taken as the taste threshold.

Studies made on adrenalectomized rats bring out the significance and possible practical uses of taste threshold investigations. Their salt threshold fell far below normal. They distinguished between salt solution and distilled water in concentrations of 0.003%, or 1 part of salt to 33,000 parts of water, while normal rats did not make this distinction until a solution of 0.055%, or 1 part of salt to 2000 parts of water, had been reached (Richter, '39).

Studies which were made on humans with a comparable choice technique between a solution and distilled water showed that humans and rats have almost the same salt thresholds. Humans distinguished salt solution from distilled water in concentrations of 0.016%, but did not recognize a salt taste until the concentration reached 0.087%, which is only slightly above the concentration at which rats showed their preference for salt solution (Richter and MacLean, '39).

As yet we have not made any studies on patients with Addison's disease or with other diseases involving destruction of the adrenal cortex. We do know, however, that such individuals, like the adrenalectomized rats, often show a very strong craving for salt (Wilkins and Richter, '40).

Similar studies were made on sucrose taste thresholds. Rats first distinguished between distilled water and sucrose solution in concentrations averaging 0.5%. Studies which were made on humans again showed very close agreement with the animal experiments. Humans first recognized a difference

between sucrose solution and distilled water in concentrations averaging 0.1%, but did not recognize a sweet taste until the concentration reached 0.41% (Richter and Campbell, '40).

In the following experiments a study was made of the rat's responses to five common sugars—maltose, glucose, sucrose, galactose, and lactose. We were interested to determine if, when the rats first distinguished between solutions of these sugars and water, they would indicate a preference or a dislike for the above carbohydrates. Also a comparison of the thresholds of these different sugars was made.

In these experiments, after the thresholds had been reached, the concentrations of the solutions were increased daily in large steps up to the point of saturation for maltose, galactose,

TABLE 1

Basal diet [1]

	gm.
Graham flour	72.5
Casein	10.0
Skim milk powder	10.0
Butter	5.0
Calcium carbonate	1.5
Sodium chloride	1.0

[1] Suggested by Dr. E. V. McCollum and used in this laboratory as a standard stock diet for all laboratory rats.

and lactose and up to 80% for glucose and sucrose. We were interested to learn how much of each of the sugars the rats take at the different concentrations, and whether they would show a preference for each of the sugars at any particular concentration.

METHODS

Previous papers contain a full description of the technique used for taste threshold experiments in rats. The rats were kept in separate cages, each of which contained two graduated inverted 100 cc. bottles and a food cup filled with a mixture made according to the formula in table 1. Fat constituted 14.1%, carbohydrate, 59.2%, and protein, 26.7% of this food mixture.

The intake from the two bottles was measured daily; body weight was recorded weekly.

For 10 to 20 days distilled water was offered in both bottles. At the end of this time, when usually the daily intake from each bottle had reached a fairly constant amount, one bottle was filled with a subliminal concentration of a sugar solution. Thereafter the concentration was increased daily in small steps until the rats definitely distinguished between the sugar solution and distilled water. From then on we increased the sugar strength in much larger steps. Table 2 gives the concentrations used in the glucose experiments and may serve to explain the time-concentration abscissae of the diagrams.

TABLE 2

Sugar concentrations used in the experiments

	%	%	%	%	%
	0.01	0.50	1.80	11.0	40.0
	0.02	0.60	2.00	13.0	45.0
	0.04	0.70	2.20	15.0	50.0
	0.06	0.80	2.40	17.0	55.0
Glucose	0.08	0.90	2.80	19.0	60.0
	0.10	1.00	3.00	21.0	65.0
	0.20	1.20	5.00	25.0	70.0
	0.30	1.40	7.00	30.0	75.0
	0.40	1.60	9.00	35.0	80.0
Maltose	From 0.01 to 50% in 24 steps				
Sucrose	From 0.20 to 80% in 28 steps				
Galactose	From 0.01 to 25% in 35 steps				
Lactose	From 0.01 to 17% in 35 steps				

The concentrations of the other sugars roughly followed this same progression up to 80% or up to their respective saturation points.

Throughout the entire experiment each rat kept its own two bottles which were marked with adhesive tape. Each bottle remained in one particular corner of the cage.

Further, in order to eliminate as far as possible any difference between the distilled water and the sugar solution, other than taste, we took the distilled water for the water bottles and the sugar solutions from the same source of

supply. The sugar solutions were watched closely for gross bacterial and fungoid contamination; and in all instances of contamination the bottles were washed with soap before autoclaving or cleaned with potassium dichromate cleaning solution when an adherent film was present. We prepared fresh sugar solutions daily.[2]

Heating in a 50°C. water bath for 15 minutes dissolved the sugars and also stabilized the mutarotations, thus avoiding differences in taste which might depend on the alpha and beta forms. The solutions were not used until they had cooled to room temperature.

Forty-four young adult male rats of approximately the same age, 65 days, were used in these experiments.

RESULTS

Preference between water and sugar solutions

At the threshold concentration the rats showed a definite preference for maltose and glucose over distilled water, just as they had shown for sucrose. Figure 1 gives the average daily intake of distilled water and glucose solution for eight rats. The ordinates give the fluid intake in cubic centimeters; the abscissae, time in days and also the concentrations of the glucose solution which were offered each day. The first 10 days give the intake records of the distilled water from the two bottles. During this time the intake remained fairly constant and approximately the same for each bottle. Seven days after the rats had a choice between glucose solution and distilled water, they began drinking more glucose solution and less water, evidently because at this time the strength of the glucose solution had reached the taste threshold. Each day thereafter, until the concentration reached 11%, the intake of the glucose solution increased at a rapid rate. As the concentration increased beyond this point, the intake of the glucose solution decreased sharply. Figures 2A and 2B show

[2] The sucrose (U.S.P.), glucose (U.S.P.) and lactose (U.S.P.) were obtained from Merck and Company, Inc.; the maltose (C.P.) and galactose (C.P.) from the Pfanstiehl Company. Due to the high cost of purified levulose, we could not use this sugar in the present study.

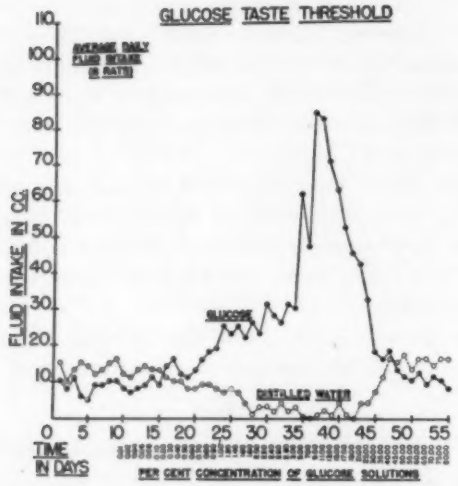

Fig. 1 Curves showing the average daily intake of distilled water and glucose solution of varying concentrations for eight rats. The ordinates give fluid intake in cubic centimeters; the abscissae, time in days and the concentrations of the glucose solutions in per cent.

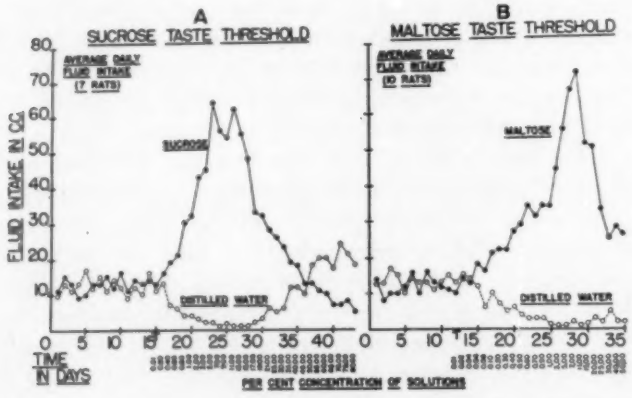

Fig. 2A Curves showing the average daily intake of distilled water and sucrose solution for seven rats.

Fig. 2B Same for maltose solution (ten rats).

the average curves for sucrose (seven rats) and maltose (ten rats) which were similar to those of glucose.

The rats showed only a mild preference for galactose over distilled water. Figure 3 gives the average curve for ten rats. The intake reached a less well-defined peak and then decreased sharply.

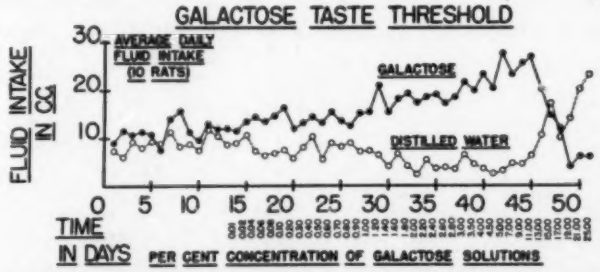

Fig. 3 Curves showing the average daily intake of distilled water and galactose solution for ten rats.

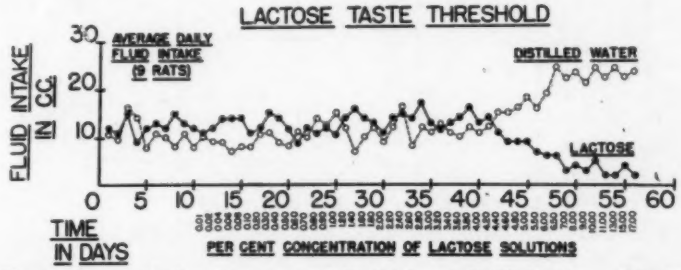

Fig. 4 Curves showing the average daily intake of distilled water and lactose solution for nine rats.

In marked contrast to the rats on the above carbohydrates, nine rats did not definitely prefer lactose to distilled water. Four rats showed a doubtful slight temporary preference (table 1). Figure 4 shows that the average intake curves for the two bottles remained almost the same up to the forty-first day, when the concentration had reached 4.8%, at which point the rats drank less of the lactose solution than of the distilled water.

Taste thresholds

The concentration at which the rats began drinking more sugar solution and less distilled water, and which we have taken as the taste threshold, was far lower for maltose than for any of the other sugars. Table 3 summarizes the results. It gives the thresholds for the individual rats and the average for each sugar. The thresholds of the ten rats on maltose averaged 0.06% and ranged from 0.04 to 0.08%. The eight

TABLE 3

Taste thresholds in per cent

MALTOSE		GLUCOSE		SUCROSE		GALACTOSE		LACTOSE		
Rat	Taste	Rat	Taste	Rat	Taste	Rat	Taste	Rat	Taste	
									(Incr.)	(Decr.)
1	0.08	1	0.2	1	0.8	1	1.8	1	...	2.8
2	0.04	2	0.1	2	0.4	2	1.8	2	1.6†	6.0
3	0.06	3	0.3	3	0.4	3	2.8	3	2.2†	5.0
4	0.08	4	0.3	4	0.8	4	1.2	4	...	4.6
5	0.06	5	0.4	5	0.1	5	1.8	5	2.2†	4.2
6	0.04	6	0.2	6	0.6	6	1.6	6	...	6.5
7	0.08	7	0.3	7	0.4	7	2.4	7	2.4†	6.5
8	0.06	8	0.2			8	0.5	8	...	3.0
9	0.06					9	1.0	9	...	4.6
10	0.08					10	1.2			
Average	0.06		0.20		0.57		1.6		2.2†	4.8
Variation	0.04–0.08		0.1–0.4		0.1–0.8		0.5–2.8		...	2.8–6.5

rats on glucose had the next lowest threshold, averaging 0.20% and ranging from 0.1 to 0.4%. Next came the threshold of the sucrose rats, averaging 0.57% and ranging from 0.1 to 0.8%; next, the threshold of the galactose rats, averaging 1.6% and ranging from 0.5 to 2.8%. Four of the nine rats on lactose increased their intake very slightly at the levels indicated on the table. All nine rats decreased their lactose intake very definitely at an average of 4.8%, and a range of variation from 2.8 to 6.5%. The points at which the sugar and distilled water curves diverged may be seen in the respective charts.

A comparison of the thresholds of these sugars, using maltose as 1, gives the group relationships indicated in table 4. We have not been able to correlate these differences with physical and chemical properties of these sugars.

TABLE 4

Taste thresholds taking maltose as unity

	%	RATING
Maltose	0.06	1
Glucose	0.20	5
Sucrose	0.57	10
Galactose	1.60	30
Lactose	2.20 (?)	40 (?)
	4.2	70

Maximum preference concentrations

The concentration at which the rats drank the largest amounts of the different sugar solutions varied less than did the taste thresholds. Table 5 summarizes the results:

TABLE 5

Maximum preference concentrations

SUGAR	PEAK CONCENTRATION	AVERAGE DAILY INTAKE OF SUGAR AT PEAK CONCENTRATION
	%	cc.
Glucose	11 (9–13)	90 (73–103)
Maltose	10 (7–11)	75 (61– 91)
Sucrose	8 (5–13)	65 (50–119)
Galactose	9 (5–13)	28 (22– 49)
Lactose	.....	15 (highest daily average at any time)

Thus, for the four sugars—glucose, maltose, sucrose and galactose—the peak concentration averaged 8.5% and ranged from 8 to 11%. The amount of the solution taken daily at the peak concentration varied through a wider range, from 90 cc. for glucose solution to 28 cc. for galactose, or to 15 cc. for the highest average reached for lactose at any time.

In considering the significance of this peak concentration, we must take into account the fact that at the peak the rats on glucose, maltose, and sucrose received with the solution from three to four times as much water as normally. It is quite possible that an excess supply of water may have stopped the rats from drinking even more of some of the higher concentrations. Against this possibility is the fact that rats

of this weight are able to excrete far larger amounts of water per day, 180 cc. and more (Richter, '38).

Amount of sugar in grams ingested at the various concentrations

We have seen that the rats drank the largest amounts of sugar solutions at concentrations of 9 to 11%. Does this mean that at this concentration they have reached their capacity for the carbohydrates? Figure 5 gives the average intake

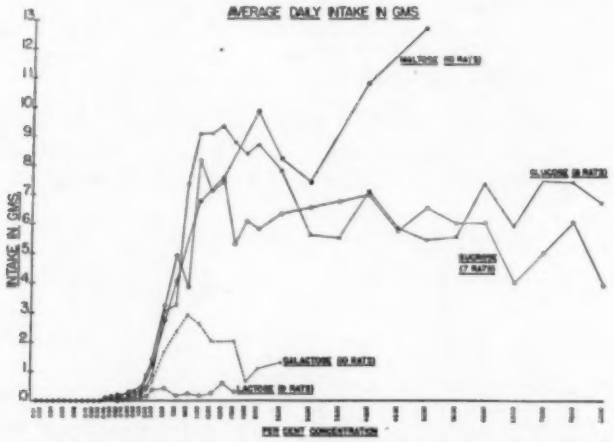

Fig. 5 Curves showing the average daily intake of the five sugars in grams with relation to the concentrations of the solutions. The ordinates give the average daily intake in grams; the abscissae give time in days and concentrations of the solutions in per cent.

in grams of the sugars at the different concentrations. From 2 to 9% the intake of maltose, glucose, and sucrose increased at approximately the same rate. With higher concentrations the intake curves began to diverge. The glucose intake reached a peak of 9.3 gm. at 15%, then decreased until it reached a level near 6 gm. at 30%, at which it remained fairly constant until the concentration reached 80%. Sucrose intake attained a peak of 8.3 gm. at 11% and decreased sharply. From 17 to 60% it remained fairly constant at a level near 6 gm. and then decreased slightly more, finally averaging

about 4 gm. at 80%. In marked contrast, the maltose intake continued to increase at a steady rate up to the saturation concentration of 50%, when it averaged 12.8 gm., over twice as much as the intake of sucrose and glucose at the same concentration. The galactose intake reached a peak of 2.7 gm. at 9% and then decreased gradually until the solution began to crystallize at 25%. The lactose intake attained a peak of 0.4 gm. at 15%.

During all of these tests the rats continued to grow at their normal rate.

DISCUSSION

The results of these experiments have shown that rats have a great appetite for maltose and a lack of appetite for, or an actual aversion to, lactose. It is conceivable that appetite or aversion may in some way depend on the constituents of the basic diet. For instance, a high lactose content may reduce or eliminate the appetite for any additional lactose; or a low maltose intake may stimulate the rats to seek more maltose. However, with its high proportion of graham flour the diet contains approximately 60% carbohydrate chiefly in the form of starch; and with its relatively small proportion of skim milk powder, 10%, or 2% lactose, the diet does not have a superabundance of lactose.

That the rats actually were able to utilize the high amounts of maltose that they ingested we know from the fact that they grew at the normal rate and remained in good health. Whether they would have continued over a longer period of time to take such large amounts of maltose was not determined in this experiment.

On the basis of the observations made over a wide range of self-selection experiments it would seem likely that the great appetite for maltose indicates that the rats are able to utilize maltose better than any of the other sugars tested and that they are least well able to utilize the lactose.

The recent experiments of Maddock, Hawkins and Holmes ('39) indicate that maltose may be utilized readily and directly without being broken down into glucose. These workers showed that in dogs and rabbits hepatectomy

abolished the normal waves of the electro-encephalogram, presumably because in the absence of the liver, some sugars can no longer be metabolized. Injections of maltose, glucose, and mannose into the carotid artery reestablished the normal waves, while lactose and galactose had no effect.

In agreement with the present observations on lactose, we have found in experiments in which rats had access to only one sugar and no other food at all that glucose gave a survival time of 57 days, sucrose 43 days, while lactose and galactose gave only 7.5 and 6.5 days, respectively. It may be that, as Schantz, Elvehjem and Hart ('38) and Schantz and Krewson ('39) have pointed out, lactose cannot be utilized in the absence of certain natural food fats. However, the McCollum diet with its 5% content of butter has an ample amount of natural fats for the utilization of larger amounts of lactose than are contained in the regular food. We must consider also that the appetite for lactose may change with age.

The taste threshold experiments brought out the surprising observation that rats have a lower threshold for maltose than for any of the other sugars. This may mean that they are able to taste it in foods in very small quantities. They recognized maltose solution in concentrations of 1 part of maltose to 2000 parts of water. Apparently, the rats distinguished galactose and lactose from water only when present in much higher concentrations.

It will be of interest to know if these thresholds can be changed by interference with the functions of the pancreas, adrenals, or pituitary. From other self-selection experiments we know that nearly all pancreatectomized rats have an aversion to sucrose. It is possible that they may be attracted to one of the other sugars.

The fact that the rats showed the maximum preference for most of the sugars at concentrations of 8 to 11% is of interest since, according to Engel ('28) and others, most drinks—coffee, tea, etc.—used by humans have an average concentration of 9% sucrose. Krogh-Jensen, working with Pauli ('25), found experimentally by actual measurements that

humans prefer sucrose solution at 9%. Engel found that at 9% all of his subjects stated that they liked sucrose solution, while with lower and higher concentrations some individuals found the taste unpleasant.

In preliminary experiments made on humans we have found that in the higher concentrations, approximately 50%, humans also had a definite preference for maltose. At 50% they actually stated that the maltose had a delicious flavor. This holds only for maltose that has been freed from the characteristic flavor of malt which gives less purified preparations such a penetrating taste. We used only the purified preparations.

SUMMARY

1. Rats distinguished between sugar solution and distilled water in the following concentrations:

	%
Maltose	0.06
Glucose	0.20
Sucrose	0.57
Galactose	1.60
Lactose	——

2. They showed the greatest preference for maltose, next for glucose and sucrose, only a slight appetite for galactose, and none for lactose.

3. The animals exhibited the greatest preference for solutions of the various sugars when offered in concentrations near 10%. This agreed closely with concentrations of sugar solutions most commonly used by humans to sweeten drinks.

4. On the basis of results of previous self-selection reports it was concluded that, when offered in purified form to adult animals, maltose may be utilized better than the other sugars, lactose least well.

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LITERATURE CITED

BERNARD, CLAUDE 1859 Lecons sur les proprietes physiologiques et les alterations pathologiques des liquides de l'organisme. Paris, Bailliere.

CANNON, W. B. 1932 The Wisdom of the Body. New York, W. W. Norton & Co., Inc.

ENGEL, RUDOLF 1928 Experimentelle Untersuchungen über die Abhängigkeit der Lust und Unlust von der Reizstärke beim Geschmackssinn. Arch. f. d. ges. Psychol., vol. 64, p. 1.

HARROP, G. A., L. J. SOFFER, R. ELLSWORTH AND J. H. TRESCHER 1933 Studies on the suprarenal cortex. III. Plasma electrolytes and electrolyte excretion during suprarenal insufficiency in the dog. J. Exp. Med., vol. 58, p. 17.

LOEB, ROBERT F. 1933 Effect of sodium chloride in treatment of a patient with Addison's disease. Proc. Soc. Exp. Biol. and Med., vol. 30, p. 808.

MADDOCK, S., J. E. HAWKINS, JR., AND E. H. HOLMES 1939 The inadequacy of substances of the "glucose cycle" for maintenance of normal cortical potentials during hypoglycemia produced by hepatectomy with abdominal evisceration. Am. J. Physiol., vol. 125, p. 551.

PAULI, R., AND A. WENZL 1925 Experimentelle und theoretische Untersuchungen zum Weber-Fechnerschen Gesetz. Arch. f. d. ges. Psychol., vol. 51, p. 399.

RICHTER, C. P. 1939 Salt taste thresholds of normal and adrenalectomized rats. Endocrinology, vol. 24, p. 367.

———— 1938 Factors determining voluntary ingestion of water in normals and in individuals with maximum diabetes insipidus. Am. J. Physiol., vol. 122, p. 668.

———— 1936 Increased salt appetite in adrenalectomized rats. Am. J. Physiol., vol. 115, p. 155.

RICHTER, C. P., AND B. BARELARE, JR. 1939 Further observations on the carbohydrate, fat, and protein appetite of vitamin B deficient rats. Am. J. Physiol., vol. 127, p. 199.

RICHTER, C. P., AND K. H. CAMPBELL 1940 Sucrose taste thresholds of rats and humans. Am. J. Physiol., vol. 128, p. 291.

RICHTER, C. P., AND J. F. ECKERT 1937 Increased calcium appetite of parathyroidectomized rats. Endocrinology, vol. 21, p. 50.

RICHTER, C. P., L. E. HOLT, JR., AND B. BARELARE, JR. 1938 Nutritional requirements for normal growth and reproduction in rats studied by the self-selection method. Am. J. Physiol., vol. 122, p. 734.

RICHTER, C. P., AND A. MACLEAN 1939 Salt taste threshold of humans. Am. J. Physiol., vol. 126, p. 1.

RICHTER, C. P., AND E. C. H. SCHMIDT, JR. 1940 Fat, carbohydrate, protein, and yeast appetite of pancreatectomized rats. (In press.)

SCHANTZ, E. J., C. A. ELVEHJEM AND E. B. HART 1938 The relation of fat to the utilization of lactose in milk. J. Biol. Chem., vol. 122, p. 381.

SCHANTZ, E. J., AND C. F. KREWSON 1939 Further studies on relation of fat to utilization of lactose in milk. Proc. Soc. Exp. Biol. and Med., vol. 42, p. 577.

WILKINS, L., AND C. P. RICHTER 1940 A great craving for salt by a child with cortico-adrenal insufficiency. J. A. M. A., vol. 114, p. 866.

THE PLANE OF INTAKE OF BEEF MUSCLE PROTEIN AS AFFECTING THE ENERGY AND THE NITROGEN METABOLISM OF THE MATURE ALBINO RAT[1]

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The influence of the plane of intake of protein in the form of casein, and as present in beef muscle, as affecting the utilization of food energy and protein by the growing albino rat having been investigated at considerable length in this laboratory (Forbes, Swift, Black and Kahlenberg, '35; Forbes, Voris, Bratzler and Wainio, '37; Forbes, Black, Thacker and Swift, '39), the purpose of the study to be discussed was to investigate the same matter with adult rats as the experimental subjects in order to learn how the plane of intake of protein affects animals of such maturity that they are no longer able to store protein in significant quantities.

Among the possible applications of the results of this series of studies, one that has been prominent in the minds of the experimenters is their bearing on the protein content of diets for human beings—growing and adult—especially at environmental temperatures above the zone of thermal neutrality; the present unit of this series, therefore, is significant in relation to mature human beings.

With the approach of summer weather, writers of newspaper health columns frequently advise restriction of the content, in the diet, of protein in general, but of meat in particular, with the idea of diminishing the heat production, and thus avoiding

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discomfort and possible prostration as a result of the overtaxing of the powers of adaptation to elevated temperatures. A question of importance, from several points of view, is whether this advice is founded on correct or on incorrect understanding.

The three published papers referred to cover, in all, five experiments. In the first and second of these studies the diets contained 10, 15, 20 and 25% of protein, respectively, this protein being almost exclusively casein; in the third experiment, also with casein, the diets contained 25, 30, 35 and 45% of protein, respectively; and in the fourth and fifth experiments the protein was mainly that of beef muscle, the diets containing 10, 25, 35 and 45% of protein, respectively.

Among the findings from these experiments with growing rats were the following: Corresponding to progressively greater protein contents of equicaloric diets, from 10% to 45%, were increased digestibility and decreased metabolizability of food energy; decrease in heat production at a diminishing rate of decrease; increase in energy of urine; increase in gain in body weight and in energy of body gain until the optimum proportion of protein in the diet was reached, and, with further increase in protein, slight decreases in rate of gain in weight, energy, nitrogen and fat, and in fat gained per gram of nitrogen gained. Also, it was shown that in the assumedly complete diets used, with growing rats as subjects, beef muscle protein does not have a greater heat stimulating effect than does casein.

The most striking observation was that in these equicaloric diets, differing as to protein content, there were not in evidence dynamic effects corresponding to the differences in protein intake.

Apparently the difference in heat production from a high-protein diet and a low-protein diet of the same gross energy value is the resultant of several factors—difference in the metabolizable energy of the diets; difference in the synthesis of food nutrients into body substance, thus leaving different amounts of nutriment available for heat production; and pos-

sible differences in the basal metabolism, and in heat increments due to the food and to the voluntary activity.

It should be realized that the difference in heat increment accompanying the difference in protein intake is not an effect of an addition of protein to the low-protein diet, but is the result of the substitution of protein for equicaloric carbohydrate and fat; and also that this difference in heat production is not a difference in the sums of the separate dynamic effects of the associated nutrients, but in the dynamic effects of the diets as entities.

With this understanding, derived from experiments on growing rats, the present experiment with mature rats was undertaken.

The principle of the body balance method, by which this investigation was conducted, is that food energy minus energy of excreta and of body gain equals heat production.

This procedure was first employed by Swift, Kahlenberg, Voris and Forbes ('34). While the measurement of heat production is indirect, the determinations on which it depends are highly consistent, and the single value for the heat produced in an experimental unit during a long period of observation possesses an accuracy and a significance as representing the total of vital activities unquestionably greater than would characterize any short-time measurement raised, by multiplication by a large number, to a value representing an equally long period.

The rats used as subjects in this investigation were chosen from individuals aged 255 to 273 days, the extremes in live weight being 337 and 410 gm. Ten quadruplets of male rats, four from each of ten litters, were selected, three from each quadruplet to be fed, and one to serve as a member of the control group which was killed and analyzed at the beginning of the experiment to give data on the initial status of those which were fed, thus providing a basis for the determination of body gains during the experiment. Actually, however, the control group contained only nine rats, the tenth having been sacrificed by error.

The experimental diets employed were similar in nature to those used in the previously published units of the general program of studies. Their composition is given in table 1.

The objective being equicaloric diets containing 10, 25 and 45% of protein, respectively, these mixtures were based on constant proportions of "Cellu flour," Osborne and Mendel salt mixture, sodium chloride, butterfat and yeast, and different proportions of dextrin, "Crisco" and dried beef muscle, the resulting diets containing, by analysis, 10.13%, 25.06% and 44.88% of protein, respectively, and 4785 calories of gross energy per gram.

TABLE 1
Make-up of diets

	DIET 1	DIET 2	DIET 3
	%	%	%
Cellu flour	4.00	4.00	4.00
Salt mixture [1]	4.00	4.00	4.00
NaCl	1.00	1.00	1.00
Butterfat	1.00	1.00	1.00
Yeast [2]	6.00	6.00	6.00
Dextrin	60.18	47.35	30.23
Beef muscle	8.58	27.94	53.77
Crisco	15.24	8.71	0.00
Total	100.00	100.00	100.00

[1] Osborne and Mendel.

[2] The yeast used was a mixture of 5 parts brewer's yeast and 1 part irradiated yeast; and carotene, in oil, was added to each diet in a quantity providing 1.4 U.S.P. units of vitamin A equivalent per gram.

All rats were given 1 week's preliminary feeding before beginning the experiment proper, with ten rats on each of the three diets, and with the controls on a mixture of equal parts of each of the three diets.

During the 10 weeks' experimental feeding the subjects were fed once a day, with triplet food control, the quantity given being determined, from day to day, by the amount of the ad libitum food consumption of the rats on the diet consumed in the smallest quantity—this being nearly always the 10% protein diet.

The average daily food consumption for the experiment as a whole was approximately 13 gm., which proved to be only slightly more than the maintenance requirement, since the average initial and final live weights were 388 and 393 gm., respectively.

At the end of the experiment the rats were killed by anaesthesia, and the bodies were comminuted, dried in vacuum, extracted with ether, ground, and analyzed in the usual manner, the entire bodies being accounted for in two samples—ether extract, and extraction residue.

The reduction of the dried and extracted bodies to a satisfactory condition for sampling and chemical analysis was accomplished by grinding several times through an "Excelsior" laboratory mill. In order to serve this exacting purpose, however, it is necessary to babbitt the interior of this mill in such manner as to fill certain spaces in which material to be ground would otherwise lodge; also the efficiency of the mill was increased by fastening a small wing of sheet metal onto the rotating shaft in such position that it agitated the material to be ground; and further, the cutting up of the dried skin, with shears, was necessary in order to prepare it for passing between the burrs of the mill. The shearing action of the interlocking teeth of the burrs of this mill serves to reduce the dried and extracted flesh, skin, hair, bones and teeth, all together, to a perfect condition for sampling and chemical analysis.

At the time of sacrificing the experimental subjects, gross examination revealed a pathological condition of the lungs of several of the rats. The specific character of this disorder was not determined, since the method of experimentation required that all parts of the bodies be accounted for by chemical analysis. The observed abnormalities are believed to have been caused by colds; however, no evidence was obtained to show that this condition appreciably affected the conclusions drawn from the results of this study.

The averages of the analytical data from the control group were as follows: live weight 373 ± 11 gm.; empty live weight

362 ± 11 gm.; ether extract 31.57 ± 2.74 gm.; air-dry extraction residue 106.2 ± 2.92 gm.; total nitrogen 12.8 ± 3.9 gm.; and total energy 751 ± 35 Calories.

In all tables the ± values signify probable errors.

The quantities of food eaten, the initial and final live weights, and the gains in empty body weight, in nitrogen and in fat are presented in table 2.

In computing these gains it was assumed that the initial composition of each of the subjects of experimentation was the same as the average composition of the nine individuals of the control group.

Since the experimental subjects were mature, but were fed virtually as much as they would eat, it is natural that they

TABLE 2

Food eaten, and gains in live weight, nitrogen and fat during 70 days

PER CENT OF PROTEIN IN DIET	FOOD EATEN, DRY MATTER	INITIAL LIVE WEIGHT	FINAL LIVE WEIGHT	GAIN IN EMPTY BODY WEIGHT	NITROGEN OF BODY GAIN	FAT GAINED
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
10.13	837	385 ± 4	391 ± 7	8 ± 3	−0.2 ± 0.1	11.53 ± 1.72
25.06	835	388 ± 3	401 ± 6	15 ± 6	0.1 ± 0.2	12.06 ± 3.76
44.88	837	390 ± 3	388 ± 7	−2 ± 6	0.1 ± 0.2	−0.18 ± 2.19

were essentially in nitrogen equilibrium. The rats on the 10% and the 25% protein diets, however, were able to gain small quantities of fat, but there was no gain of fat from the 45% protein diet, because of its lower metabolizability.

In table 3 is represented the distribution of food energy as affected by the plane of protein intake.

The positive balance of protein is shown to have been insignificantly small from all three diets, and a slight body gain in energy from the two lower-protein diets is shown to have been in the form of fat.

The progressive replacement of dextrin and Crisco in the 10% protein diet with dried beef muscle, for the production of equicaloric diets containing from 10% to 45% of protein, resulted in increases in the digestible energy of the diets

from 3722 Calories to 3808 Calories, as totals per rat for the 70-day experimental period; but the coincident increase in urinary energy more than offset this increase in digestible energy, with the result that in the order of the increase in dietary protein from 10% to 45%, there was a decrease in the metabolizable energy of the diets from 3619 Calories to 3407 Calories; and accompanying this decrease in metabolizable energy was a decrease in heat production from 3505 Calories to 3402 Calories, for the entire experimental period.

The odds that the heat from the 45% protein diet was less than from the 10% protein diet were 147 to 1. The odds that

TABLE 3

Distribution of total food energy as affected by the plane of protein intake

PER CENT OF PROTEIN IN DIET	10.13	25.06	44.88
Energy value in calories:			
Food	4220	4220	4220
Feces	498 ± 8[1]	403 ± 5	413 ± 6
Digested material	3722 ± 48	3817 ± 50	3808 ± 50
Urine	103 ± 1	237 ± 3	401 ± 4
Metabolized material	3619 ± 47	3579 ± 48	3407 ± 46
Body gain: total	113 ± 18	124 ± 36	5 ± 25
as protein	9 ± 4	14 ± 6	12 ± 8
as fat	104 ± 16	110 ± 35	− 6 ± 21
Heat production	3505 ± 36	3456 ± 26	3402 ± 43

[1] All ± values signify probable errors.

the 25% protein diet produced less heat than the 10% protein diet, and that the 45% protein diet produced less heat than the 25% protein diet, however, were only 5 to 1, and 4 to 1, respectively, and, therefore, were not significant. These odds were computed by "Student's" method.

While this decrease in heat production is conceivably the resultant of several contributing factors, prominent among these is the coincident decrease in the metabolizable energy of the diets, in harmony with the fact that whereas protein has a gross energy value of about 5.7 Calories per gram, it has a value of only about 4.2 Calories per gram to produce heat in

the body, the metabolizable energy of carbohydrate and fat being more nearly as great as their gross energy values.

The percentages of the metabolizable energy that were expressed as heat were 96.8 ± 0.5, 96.6 ± 1.0 and 99.9 ± 0.8, respectively, in the order of the increasing protein contents of the diets. Thus, though there was a slight decrease in heat production in the order of the increase of dietary protein, these values constituted a slight increase in heat as a percentage of the metabolizable energy.

This finding is not necessarily out of harmony with the measurements of the specific dynamic effects of nutrients which have been published from this laboratory, since the experimental backgrounds in these two heat measurements have differed so radically that the results are not of the same significance.

Among a number of papers reporting specific dynamic effects of protein, a recent one by Forbes, Bratzler, Thacker and Marcy ('39) is of interest. In this study the dynamic effect of protein was determined by superimposing it upon a basal, maintenance diet, in 6-hour periods of measurement in the absence of voluntary activity; while in the present study the protein was introduced into the diet not by superimposition but by substitution for an equicaloric quantity of nonnitrogenous nutriment—this last having a higher metabolizable energy value than the protein introduced—the heat measurement representing 70 days' feeding under normal conditions of activity. It would be impossible to say what the consequences of this difference in conditions of experimentation should be in relation to the differences in heat production ascribable to the protein.

The lack of complete agreement between the very small values for grams of gain of protein and of fat, as given in table 2, and the similarly small values for calories of gain as protein and as fat, as given in table 3, results from the fact that these two sets of values were determined independently, those in table 2 as results of Kjeldahl determinations of nitrogen and of ether extract determinations of fat, and those in

table 3 as results of bomb calorimeter determinations of the energy of the ether extract and of the extraction residue, which is designated protein.

Referring to table 4, the apparent digestibility of the food nitrogen varied in the same order as the intake, but not in the same proportion, the digestibility of the protein of the 10% protein ration being 83.1%, and of the 45% protein ration 92.9%—this difference presumably signifying, at least in part, increased intake in excess of the amount eliminated as products of endogenous metabolism.

TABLE 4

Distribution of food nitrogen as affected by the plane of protein intake

PROTEIN IN THE DIET IN PER CENT	10.13		25.06		44.88	
Nitrogen:						
Food in grams	14.3		35.4		63.3	
Feces in grams	2.4	± 0.0 [1]	3.3	± 0.1	4.5	± 0.1
Digested: In grams	11.9		32.1		58.8	
In per cent of food N	83.2	± 0.2	90.7	± 0.1	92.9	± 0.1
Urinary: In grams	10.7		30.6		57.6	
In per cent of food N	74.8	± 0.8	86.4	± 0.3	91.0	± 0.4
Gain of nitrogen	− 0.2	± 0.1	0.1	± 0.2	0.1	± 0.2
Urinary nitrogen per calorie of urinary energy	0.104	± 0.001	0.129	± 0.001	0.144	± 0.001

[1] All ± values signify probable errors.

The fecal outgo of nitrogen was remarkably low, and the urinary nitrogen very high, the balance being approximate equilibrium.

The material increase in urinary nitrogen per calorie of urinary energy accompanying the increase in dietary protein from 10% to 45% is understood to signify an increasing proportion of the nitrogenous outgo in the form of urea.

The percentages of the heat production derived from the oxidation of protein were nearly the same as the percentages of protein in the three diets, namely, 8.1, 23.5, and 44.9.

In relation to the accounting for the food nitrogen in the products derived therefrom, the amounts by which the nitrogen intake per rat, during the 70-day experiment, exceeded the

nitrogen of the feces, the urine and the body gain were 1.41, 1.31, and 1.19 gm., for the 10%, the 25% and the 45% protein diets, respectively, these quantities being equivalent to 10%, 4% and 2%, respectively, of the food nitrogen.

These nitrogen losses did not represent scattered food, because food was not scattered in significant quantities, and because the relative quantities of nitrogen lost were not in accord with the nitrogen contents of the diets. Neither were these losses of nitrogen in the form of urinary ammonia, because the quantities lost differed greatly in nitrogen content, and also because the quantities of these losses of nitrogen differed in the reverse order from that of the nitrogen contents of the diets. Moreover, these losses could not have been in feces, because they were collected with accuracy; nor is it believed that they represent error in the determination of the body gain of nitrogen—since a body gain in this constituent was neither expected nor found.

By exclusion, therefore, it is concluded that these losses of nitrogen represented shed hair and scurf, which were present in considerable quantities with these mature rats, but were not collected and analyzed.

The differences between the amounts of nitrogen not accounted for, however, would not represent significant amounts of energy, in relation to the total heat production, if these losses, as assumed, represented hair and scurf, because the ratio of energy to nitrogen in hair is materially less than the equivalent value representing the diets.

By way of recapitulation of the six experiments of which the present one is the last, in the first five the subjects were growing, and in the last, mature; in the first three experiments the principal protein of the diets was casein, and in the last three, beef muscle; the range of protein contents of the diets in the first two experiments was 10% to 25%, in the third, 25% to 45%, and in the fourth, fifth and sixth, 10% to 45%, though in each experiment all diets had the same gross energy content; in all experiments there was decrease in the heat production of the subjects in the order of the increasing protein contents of

the diets, though these factors were not closely proportional the one to the other; the body gain of energy increased with increasing protein contents of the diets until the optimum was reached, and then decreased in the order of further increase in the protein contents of the diets; determinations of final basal metabolism were made in some of the experiments, but the systematic factoring of the heat production into basal metabolism, heat increment due to the diet, and heat increment due to voluntary activity was impracticable in connection with the experimental procedure employed and was not attempted.

In relation to the cause of the decrease in heat production in the order of the increasing protein contents of the diets—the metabolizable energy obviously dominates this function in the sense that no energy can be expressed as heat which is not metabolizable, but the utilization of metabolizable energy for body gain prevents its expression as heat, and in four of the experiments among the six the decrease in heat production in the order of increasing protein contents of the diets exceeded the accompanying decrease in metabolizable energy. In these four instances among the six, therefore, the metabolizable energy did not exert a clear dominance over the heat production.

In the light of the results of this investigation as a whole, it is suggested that in relation either to growing or to mature human beings it would be ineffective to diminish the proportion of protein to nonprotein material in the diet as a means of minimizing heat production during excessively warm weather; and that the logical procedures for the accomplishment of this purpose would be to avoid unnecessary activity, to be conservative as to the total quantity of food eaten, and to keep the caloric value of the food relatively low by increasing the proportions, in the diet, of watery foods such as fruits and vegetables.

In regard to fat in the hot-weather diet, conservatism in the intake of fat is desirable because of its high caloric value, but the usual fat content of the diet should not be decreased without consideration of the amount of vitamin A that would remain.

SUMMARY

The effects of the plane of protein intake on the utilization of the food protein and energy of equicaloric diets containing 10%, 25% and 45% of protein, respectively, were investigated in a 70-day balance experiment with mature albino rats as subjects.

The rats remained in approximate equilibrium with respect to live weight and to the nitrogen content of their bodies.

The digestibility of the protein of the diets increased in the order of the increase in dietary protein; and the digested nitrogen and the urinary nitrogen were essentially the same values.

The metabolizable energy diminished in the order of the increasing protein contents of the diets, primarily because the increase in the outgo of urinary energy more than counterbalanced the coincident decrease in feces energy, from the two higher protein as compared with the lowest protein diet.

The heat production diminished, slightly, in the order of the increase in protein in the diets, this diminishing heat production being equivalent to a slightly increasing heat in relation to the metabolizable energy.

Urinary nitrogen per calorie of urinary energy increased in the same order as the increase in dietary protein.

The bearing of the results in relation to human diet is pointed out.

LITERATURE CITED

FORBES, E. B., R. W. SWIFT, ALEX BLACK AND O. J. KAHLENBERG 1935 The utilization of energy producing nutriment and protein as affected by individual nutrient deficiencies. III. The effects of the plane of protein intake. J. Nutrition, vol. 10, pp. 461–479.

FORBES, E. B., LEROY VORIS, J. W. BRATZLER AND WALTER WAINIO 1937 The utilization of energy producing nutriment and protein as affected by the plane of protein intake. J. Nutrition, vol. 15, pp. 285–307.

FORBES, E. B., ALEX BLACK, EDWARD J. THACKER AND R. W. SWIFT 1939 The utilization of energy producing nutriment and protein as affected by the level of intake of beef muscle protein. J. Nutrition, vol. 18, pp. 47–56.

FORBES, E. B., JOHN W. BRATZLER, EDWARD J. THACKER AND L. F. MARCY 1939 Dynamic effects and net energy values of protein, carbohydrate and fat. J. Nutrition, vol. 18, pp. 57–70.

SWIFT, R. W., O. J. KAHLENBERG, LEROY VORIS AND E. B. FORBES 1934 The utilization of energy producing nutriment and protein as affected by individual nutrient deficiencies. I. The effects of cystine deficiency. J. Nutrition, vol. 8, pp. 197–219.

HYPERALIMENTATION IN NORMAL ANIMALS PRODUCED BY PROTAMINE INSULIN[1]

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THREE FIGURES

(Received for publication February 6, 1940)

Early in its history insulin was used in an attempt to improve the nutrition of non-diabetic patients (Pitfield, '23; Marriott, '24). It was regarded as stimulating the appetite thus leading to a higher caloric intake of food. It has been used for this purpose by many although proof of its efficacy was lacking. The reason for this is because of the many factors involved in such clinical observations, but the careful and well controlled observations of Freyberg ('35) are particularly interesting. His investigation failed to support the prevalent opinion that insulin has any significant influence upon the appetite. That ordinary insulin has no influence on body weight of rabbits has also been reported as the conclusion from experiments by Long and Bischoff ('30). The rat more closely resembles man in its dietary habits and the nature of its gastro-intestinal tract than does the rabbit. Because of the favorable reports in man it appeared desirable to repeat this study in the rat.

REGULAR INSULIN[2]

The first experiments were carried out in 1929 with the regular commercial insulin then available in a strength of 40

[1] Preliminary reports of the experiments described here were given before the Society for Experimental Biology and Medicine (Proc. Soc. Exp. Biol. and Med., vol. 36, p. 406, 1937) and the meeting of the American Association for the Advancement of Science in Denver, June, 1937.

[2] The regular insulin supplied to us in 1930 and the protamine zinc insulin used in the first part of the experiments with this product were furnished by Eli Lilly and Company of Indianapolis to whom we are indebted. Additional supplies with which to complete the work were provided by E. R. Squibb and Sons.

units per cubic centimeter. Either 3 units or 10 units were given in the morning. Results in three individual rats are charted in figure 1. They are typical of all of our data obtained with regular insulin. The diet was supplied ad libitum and was composed of powdered commercial casein 25, starch 40, lard 10, butterfat 15, tested dried brewers' yeast 5 and Osborne and Mendel's standard salt mixture 5. There is no indication that either the food intake or the body weight was augmented as a result of the administration of regular insulin. This confirms the conclusions that have been reached in studies with

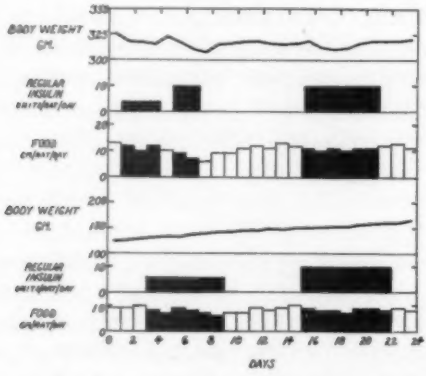

Fig. 1 Experiments illustrating the failure of regular insulin to influence the food intake or body weight of the rat. The first experiment is from data obtained on a single rat while the second represents the averages from a group of three.

the rabbit (Long and Bischoff, '30). In contrast to these results Macleod et al. ('30) concluded from experiments on pigs that "insulin had only a very slight accelerating effect on the rate of increase in weight . . . ." but an examination of their data gives no support whatever to even this questionable positive conclusion. Maclagan ('37) has noted a slight augmentation of the appetite of rabbits with insulin which was presumably of the ordinary type but this was only over a period of a few hours without any evidence that it could be continued from day to day by insulin administration.

PROTAMINE ZINC INSULIN

This slowly absorbed insulin preparation was given subcutaneously in a strength of 40 units per cubic centimeter. In every case the use of this insulin in substantial doses led to an increased appetite (fig. 2). In some cases the increase in food intake was tremendous (table 1). The excess food was largely stored in the form of fat. The relation of the weight increase

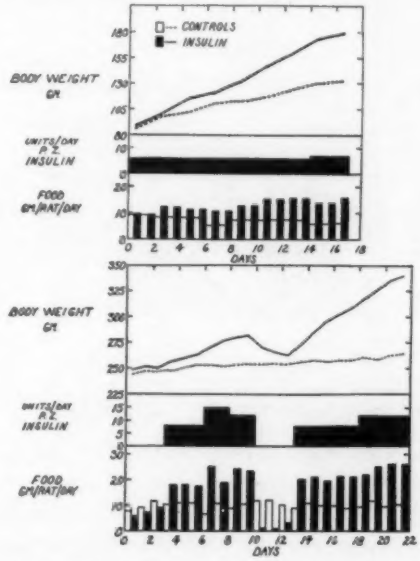

Fig. 2 Typical examples of the marked effect of protamine zinc insulin on the food intake and body weight of the rat. In both experiments the figures are averages for groups of four rats.

to the excess food consumption of comparable control rats shows that no considerable proportion of the excess calories is dissipated in an increased metabolism. There would naturally be the higher specific dynamic action of more food. We have no data on the metabolic rate. One point of interest was the 5 to 20% increase in the weight of the intestinal tract depending on the extent of the increased food intake.

APPETITE AND BLOOD SUGAR

Hypoglycemia or a more moderate reduction in the blood sugar level appears to be the chief factor in the weight gain due to protamine insulin. Frequent blood sugar determinations are not feasible in the rat but the data in figure 3 show a very definite inverse relation between the blood sugar level and the food intake in the rabbit. The food consisted of ordinary alfalfa hay and oat commercial rabbit pellets. It is true,

TABLE 1

The increase in food intake and body weight after protamine insulin

EXP. NO.	NUMBER OF RATS IN EACH GROUP	SEX	PERIOD IN DAYS	CONTROL AVERAGES				PROTAMINE INSULIN TREATED AVERAGES					INCREASE PER DAY DUE TO PROTAMINE INSULIN	
				Initial body weight	Final body weight	Increase in body weight per day	Food intake per day	Protamine insulin per rat per day	Initial body weight	Final body weight	Increase in body weight per day	Food intake per day	Food intake	Body weight
				gm.	*gm.*	*gm.*	*gm.*	*units*[1]	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	4	♂	8	57	75	2.25	6.1	2	60	80	2.50	8.0	1.9	0.25
2	5	♂	11	239	243	0.40	12.0	4	242	274	2.00	19.0	7.0	1.60
3	6	♂	20	240	261	1.05	11.2	10	242	308	3.30	16.8	5.6	2.25
4	3	♂	15	94	161	4.45	9.9	6	97	202	7.00	13.8	3.9	2.55
5	4	♀	15	86	132	3.07	7.6	6	89	178	6.00	12.6	5.0	2.93
6	4	♂	4	98	113	3.75	9.5	8	99	126	6.75	13.5	4.0	3.00
7	4	♀	4	88	101	3.25	8.1	8	92	118	6.50	13.1	5.0	3.25
8	6	♀	5	159	163	0.80	8.0	8	162	183	4.20	12.5	4.5	3.40
9	4	♀	3	204	206	0.66	9.3	12	202	227	8.33	10.1	10.8	7.67

[1] The protamine insulin dose was always divided, half of it being given at either end of the day.

of course, that the concentration of insulin in the organism is probably the highest when the blood sugar is the lowest and that insulin concentration might be the factor which governs the appetite of these animals. Other observations make this seem unlikely. It might be mentioned that the rabbit is not a very good animal for the type of experiment in which we are interested here and can never be made to gain much weight with insulin administration. The reason for this is the low energy value of its food when calories per unit volume are

considered. Rats given a very bulky diet gain slightly at first and then die in hypoglycemia because they are unable to eat enough.

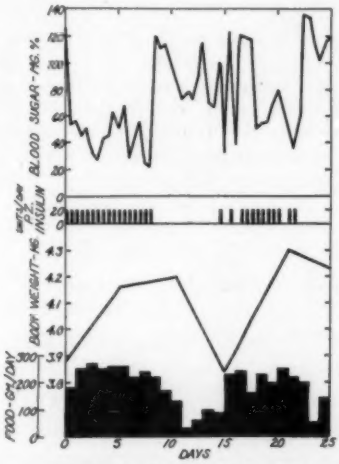

Fig. 3 The influence of protamine insulin injections upon the blood sugar level, food intake and body weight in a female rabbit. This demonstrates the typical inverse relation between the blood sugar level and the food intake.

PROTAMINE INSULIN ACTION AND ADRENAL MEDULLA

The hypoglycemia produced by insulin induces an increased secretion of epinephrine (Cannon, McIver and Bliss, '24). This mobilizes sugar from the liver and aids in counteracting the hypoglycemia. The increase in the blood sugar level which normal rats show in response to emotional excitation does not occur if the adrenal medullas have been destroyed by enucleation (Harris and Ingle, '37). If the lowered blood sugar level rather than the increased insulin concentration is the factor governing the increased appetite after protamine zinc insulin, the weight gain should be greater when insulin is given after enucleation of the adrenal medullas. It can be seen from table 2 that protamine insulin is more effective under such circumstances. The medullas were enucleated and a month allowed for the cortical tissue to regenerate before this experiment

was carried out. The insulin treated rats without their adrenal medullas had lower blood sugars than the others and had a greater tendency to die in hypoglycemia than control rats receiving the same amount of insulin.

TABLE 2

Influence of removing the adrenal medullas on the weight increase due to protamine insulin. The dose of insulin was the same for every rat. They were given 5 units twice daily for a period of 20 days. Each figure is an average for a group of six rats

GROUP	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	WEIGHT INCREASE	
	gm.	*gm.*	*gm.*	%
Male controls	253	330	77	30
Males with medullas removed	257	380	123	48
Female controls	197	265	68	35
Females with medullas removed	194	284	90	46

DISCUSSION

We cannot be absolutely certain at the present time whether the increased appetite which results from protamine insulin administration is due to the reduction in the blood sugar level or the concentration of insulin circulating in the organism, although we favor the former possibility. Assuming the decrease in the blood sugar level to be the cause, there is the question of whether its action is peripheral, through an increase in gastric hunger contractions, or central by its influence on a possible appetite center in the brain. The work of Bulatao and Carlson ('24), who found that gastric motility can be increased by depressing the blood sugar with insulin and abolished by the administration of glucose, would point to direct action on the stomach but Mulinos ('33) could find no relation between gastric motility and normal spontaneous variations in the blood sugar. It is likely that the variations under ordinary conditions would be too small to give a satisfactory correlation.

We indicated in our introduction that although ordinary insulin has never been proved to have any influence on the appetite it has often been used to improve the food intake in

man. This subject is well reviewed by Marlow and Callaway ('38). They found that unlike ordinary insulin the slow acting protamine zinc insulin was really efficacious in increasing the appetite and causing a gain in weight. The action of ordinary insulin on the blood sugar is apparently too transient in the fed organism to influence the blood sugar level for an appreciable length of time. The slowly absorbed protamine compound is much more efficacious in the rat than in man insofar as augmenting the food intake is concerned. Emotional inhibition of gastric motility and hence appetite is undoubtedly of greater importance in the case of man than the rat.

The doses of insulin which were used are not very great when we consider the pharmacology of this agent in the rat, but transferred to other species on a body size basis these doses might be enormous. This is true in the case of humans where an infinitely smaller dose of protamine zinc insulin is necessary to reduce the blood sugar level enough to influence the food intake (Marlow and Callaway, '38). We desire to point this out because of the well known dangers of hypoglycemia. Even though there may be apparent recovery from hypoglycemic symptoms a reduction in the blood sugar level for any extended period of time is very apt to lead to permanent damage to the brain (Sherrill and MacKay, '39).

SUMMARY

Regular insulin in doses of 1 to 5 units per 100 gm. body weight per day had no measurable effect upon the food intake or body weight of albino rats. The slowly absorbed protamine zinc insulin in similar doses had a remarkable influence upon the appetite. The food intake could be more than doubled in this manner leading to large increases in body weight.

The chief factor leading to the increased appetite appeared to be the decrease in the blood sugar level, for an inverse relationship between the latter and the food intake was demonstrated in the rabbit. Furthermore removal of the adrenal medullas, thus preventing the secretion of epinephrine when stimulated by hypoglycemia, and the ensuing blood sugar-

raising effect led to greater weight gains from a given dose of insulin.

LITERATURE CITED

BULATAO, E., AND A. J. CARLSON 1924 Contributions to physiology of stomach; influence of experimental changes in blood sugar level on gastric hunger contractions. Amer. J. Physiol., vol. 69, p. 107.

CANNON, W. B., M. A. McIVER AND S. W. BLISS 1924 Studies on conditions of activity in endocrine glands; sympathetic and adrenal mechanism for mobilizing sugar in hypoglycemia. Amer. J. Physiol., vol. 69, p. 46.

FREYBERG, R. H. 1935 Study of value of insulin in under-nutrition. Am. J. Med. Sci., vol. 190, p. 28.

HARRIS, R. E., AND D. J. INGLE 1937 Influence of destruction of adrenal medulla on emotional hyperglycemia in rats. Amer. J. Physiol., vol. 120, p. 420.

LONG, M. L., AND F. BISCHOFF 1930 Effect of insulin upon body weight of rabbit. J. Nutrition, vol. 2, p. 245.

MACLAGAN, N. F. 1937 Role of appetite in control of body weight. J. Physiol., vol. 90, p. 385.

MACLEOD, J. J. R., H. E. MAGEE AND W. MIDDLETON 1930 Insulin and increase in weight of young animals. Biochem. J., vol. 24, p. 615.

MARLOW, A. A., AND J. W. CALLAWAY 1938 The use of protamine zinc insulin in undernutrition. Bull. Scripps Metabolic Clinic, vol. 1, p. 25.

MARRIOTT, W. McK. 1924 Food requirements of malnourished infants with a note on the use of insulin. J. Am. Med. Assoc., vol. 83, p. 600.

MULINOS, M. G. 1933 Gastric hunger mechanism; influence of experimental alterations in blood sugar concentration on gastric hunger contractions. Amer. J. Physiol., vol. 104, p. 371.

PITFIELD, R. L. 1923 Insulin in infantile inanition. New York M. J., vol. 118, p. 217.

SHERRILL, J. W., AND E. M. MacKAY 1939 Deleterious effects of experimental protamine insulin shock. Arch. Int. Med., vol. 64, p. 907.

INTESTINAL ABSORPTION OF VITAMIN A IN THE NORMAL RAT [1]

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Evidence is accumulating which indicates that a higher conversion factor must be used for converting the extinction coefficient, $E^{1\%}_{1\,cm.}$ at 328 millimicrons, of esters of vitamin A to biological (International or U.S.P.) units than for vitamin A alcohol (Morgan, Edisbury and Morton, '35; Barthen and Leonard, '37; Moll and Reid, '39; Koehn and Sherman, '40). Attempts to establish a sound physiological basis for this difference have not been strikingly successful. It occurred to us that as a first step toward an understanding of this phenomenon, information as to the mechanism of vitamin A absorption from the intestine was needed. The study reported herewith was designed, primarily, to determine whether vitamin A esters, like other esters of the fatty acids, are hydrolyzed by the intestinal enzymes prior to absorption as well as to indicate the state of the vitamin in the gut wall during absorption.

EXPERIMENTAL

The general plan of the experiment consisted in setting up comparable groups of rats which were killed at stated intervals after the administration of a known dose of vitamin A ester. The residual intestinal contents, as well as the gut walls, were analyzed for total vitamin A and for percentage of vitamin

[1] This paper was read before the thirty-fourth annual meeting of the American Society of Biological Chemists, Inc., at New Orleans, March 15, 1940.

A alcohol. The extracts from the wall were also analyzed for free fatty acid.

PROCEDURE

Thirty-six young, adult, female rats (Wistar strain) were divided into nine groups of four each. The animals were 90 days old, averaged 145 gm. in weight and were 16 hours post-absorptive at the time of the experiment. Each rat was given a dose of 54,160 U.S.P. units of vitamin A as distilled ester concentrate from fish liver oil dissolved in 1.0 cc. of corn oil [2] by stomach tube. The free alcohol content of the preparation was less than 1%. At stated intervals after the injection of the dose, the animals in each group were etherized and the abdominal cavity exposed by a mid-line incision from the costal margin to the perineum. The gastro-intestinal tract, from the esophagus to the ileo-cecal junction, was securely ligated and removed intact. The large intestine was not taken. As much of the mesenteric fat as possible was stripped from the intestine although the removal was not absolutely complete. The contents of the stomach and intestine were washed out with several successive 5 cc. portions of normal saline which was introduced into the stomach with a syringe and carefully propelled through the length of the gut by stripping between the fingers. Gut contents and washings from the four rats in each group were pooled and extracted three times with ethyl ether in separatory funnels. The ether extracts were dried over anhydrous sodium sulfate and the total lipid analyzed after the removal of the solvent under a stream of nitrogen on the steam bath. The results are summarized in the first part of table 1. The per cent of vitamin A alcohol was determined by the technique of analytical distillation (Hickman, '36, '37). The distillate fractions were taken at 10° intervals from 90 to 240°C. and at pressures ranging from 4.0 to 1.9 μ (Parani gauge attached to still chamber). Under the conditions of distillation employed, vitamin A alcohol comes over below 150° while the

[2] The commercial corn oil, Mazola, was used.

peak of the ester elimination curve occurs between 200 and 210°. The alcohol: ester ratio of the sample is easily calculated from the areas under their respective maxima of the elimination curve.

The pooled gastro-intestinal tissue from each group was minced with scissors, ground with sand, transferred quantitatively to Soxhlet thimbles and extracted in hot-vapor percolators for 8 hours with 95% alcohol and then for a similar

TABLE 1

Recovery of vitamin A from gastrointestinal contents and tissues after administration of 216,600 U.S.P. units (total) to groups of four post-absorptive rats

GROUP	TIME AFTER DOSING	TOTAL LIPID	TOTAL VITAMIN RECOVERED		RECOVERY IN DISTILLATES		
			Per gram of lipid	Of total fed	VITAMIN DISTILLED [1]	Total	As vitamin A alcohol
				From intestinal contents			
	minutes	*gm.*	*units*	%	*units*	%	%
1	80	1.95	44,500	40.1	84,700	110.5	4.36
2	119	1.94	49,200	44.1	93,100	88.3	7.26
3	160	1.57	58,200	42.3	87,800	87.2	8.26
4	191	2.05	57,350	54.4	112,300	86.7	14.10
5	220	1.91	60,000	53.1	110,000	95.5	12.16
6	248	1.44	62,250	41.5	87,100	91.7	10.42
7	277	1.06	77,900	39.3	82,100	80.1	14.10
8	337	1.32	63,100	38.6	79,300	112.7	16.28
9	398	0.87	75,200	30.3	62,700	101.5	16.20
				From G.I. tissue			
1	80	1.97	3650				
2	119	1.48	1750				
3	160	0.95	3420		22,400	88.6	59.20
4	191	1.42	4140		(free fatty acids = 39.2%)		
5	220	1.59	3590				
6	248	1.17	4650				
7	277	1.72	4520		28,100	96.4	81.90
8	337	2.39	3250		(free fatty acids = 48.20%)		
9	398	1.81	5140				

[1] Prior to distillation, all samples were made up in a definite amount of constant yield diluent oil. The values in this column represent the total vitamin A actually placed in the still as determined by direct analysis of the diluted sample. Since a small but accurately known loss occurs on transfer of the sample to the still reservoir, these values are slightly less than the total vitamin recovered as indicated by the product of the figures in columns 3 and 4.

period with ethyl ether. The combined alcohol-ether extracts were evaporated under nitrogen on the steam bath to a watery residue which was transferred to separatory funnels and reextracted with petroleum ether (redistilled, b.p. 38–45°). The solvent was again removed under nitrogen and the total lipid weighed and analyzed for total vitamin A. The results are given in the second part of table 1. Due to the small amounts of vitamin found in the individual group extracts, it was necessary to combine groups 1 to 5 and 6 to 9 in order to carry out the analytical distillation for the separation of the ester and alcohol. Thus, only two composite values for vitamin A alcohol of the G.I. tissues were obtained corresponding to the first and second halves of the experimental period, respectively. They are, however, of sufficient significance to justify including them. Free fatty acid content of the total lipid of the two composite samples was determined by titration with alcoholic NaOH. The values are indicated in the table.

It should be added here that the total vitamin A values for the lipid of the gut contents were determined by ultra-violet absorption ($E^{1\%}_{1\ cm.}$ at 328 millimicrons) using the Hilger medium quartz spectrograph (E-498) in conjunction with the Spekker photometer. The values for the tissue lipids and for the distillate fractions were obtained by means of the antimony trichloride reaction using the Evelyn photo-electric colorimeter (Dann and Evelyn, '38). It has been our experience that the two methods are consistent to about $\pm 15\%$.

DISCUSSION

Data furnished by studies of this type are of interest with respect to the mechanism of vitamin A absorption from the gut. The difference between biological conversion factors for ester and alcohol has been mentioned. These data indicate that vitamin A esters behave as do other esters of the fatty acids in the intestinal tract and show that they are certainly capable of hydrolysis by the enzymes of the intestinal juice. The additional demonstration that during the height of ab-

sorption, the vitamin within the gut wall exists mainly in the unesterified form can be taken as a potent argument against any assumption that the esters, as such, are directly absorbed from the lumen into the blood. The fact that vitamin A has been largely reesterified by the time it appears in the circulating blood (Clausen et al., '40), however, is quite consistent with generally accepted views of fat metabolism.

Lovern and his associates ('39, '39 a) have indicated the possibility that vitamin A may play an important role as a transporting agent for fatty acids across the gut wall in the halibut. Even though this may be true for certain fish, and the idea presents an interesting reason for the occurrence of large amounts of this substance in these species, the evidence at present does not constitute any substantial argument for the existence of such a mechanism in the higher mammals.

In this connection, it is of interest to point out that the occurrence of free fatty acids in the lipid extracts of intestinal mucosa has long been recognized but never satisfactorily explained. Sinclair ('28) found that as much as 36% of the acetone soluble material from the post absorptive cat mucosa was free fatty acid. During fat absorption he found somewhat lower values. About one-third of this amount could be accounted for by the acids liberated from neutral fat by intestinal hydrolysis and simply adherent to the mucosa from which it was not removed by washing. Our finding of a titratable acidity equivalent to nearly 50% free fatty acid in the total lipid from the whole intestine cannot be accounted for on this basis alone. Neither does it seem reasonable that phospholipid breakdown, with its consequent liberation of free acid, was excessive since extraction procedures such as we employed have long been used in the isolation of phospholipid. It is well known that the phospholipids are among the least stable of the tissue lipids and it has been suggested by Bloor ('24), McLean and McLean ('27) and others that a substantial part of the free fatty acid found in these extracts may have arisen from the splitting of these

compounds. Sinclair's data show that phospholipid amounts to between 35 and 45% of the total lipid of the intestinal mucosa. These values were determined as acetone insoluble fractions of the usual alcohol-ether extracts. Theoretically, fatty acids make up about 70% of the phospholipid molecule although direct analysis usually yields values between 50 and 60%. Thus, if the entire phospholipid of the intestinal mucosa were hydrolyzed during extraction, a circumstance which obviously does not occur, fatty acid from this source could not amount to more than 25% of the total lipid.

It appears to be worth noting that administered fat is absorbed from the intestine more rapidly than vitamin A since, as shown in table 1, the lipid content of the lumen becomes relatively richer in the vitamin as absorption progresses. This fact also argues against a major role for vitamin A in fat absorption. From a purely quantitative point of view, the normal occurrence of only negligible amounts of vitamin A in the gut wall of the rat (Baumann, Riising and Steenbock, '34), in comparison with relatively large amounts of fatty acid, may be considered as evidence in the same direction.

SUMMARY

Vitamin A, in the form of the naturally occurring esters of fish liver oil, has been administered to post absorptive rats and the per cent of free alcohol in the lumen and in the gut wall determined at varying time intervals using the technique of analytical distillation. Of the total vitamin A recovered, it was found that the per cent of alcohol increased from 4.36 at 80 minutes to 16.20 at 400 minutes while the alcohol in the gut wall increased from 59.2% at 220 minutes to 81.9% at 400 minutes. These data indicate that vitamin A esters behave as do other esters of the fatty acids in the intestinal tract in that they are hydrolyzed by the enzymes present there and, further, that during the height of absorption, the vitamin exists in the gut wall chiefly as the alcohol.

ACKNOWLEDGMENTS

We wish to express our gratitude to Dr. W. R. Bloor and the Department of Biochemistry for making available the animal facilities which made this study possible. We are indebted to Mr. Milton Joffe for technical assistance in preparing the lipid extracts.

LITERATURE CITED

BARTHEN, CHARLES L., AND CLIFFORD S. LEONARD 1937 A comparison of the spectrophotometric and biological assays for vitamin A. J. Am. Pharm. Assoc., vol. 26, p. 515.

BAUMANN, C. A., BLANCHE M. RHISING AND H. STEENBOCK 1934 Fat soluble vitamins. XLII. Absorption and storage of vitamin A in the rat. J. Biol. Chem., vol. 107, p. 705.

BLOOR, W. R. 1924 The fatty acids of blood plasma. II. The distribution of the unsaturated fatty acids. J. Biol. Chem., vol. 59, p. 543.

CLAUSEN, S. W., W. S. BAUM, J. O. RYDEN, B. B. BREESE AND A. B. MCCOORD 1940 Determination of free and esterified vitamin A in mixtures. Am. Chem. Soc., Abstracts, 99th Meeting. Also, personal communication.

DANN, WILLIAM J., AND KENNETH A. EVELYN 1938 The determination of vitamin A with the photoelectric colorimeter. Biochem. J., vol. 32, p. 1008.

HICKMAN, K. C. D. 1936 The identification of vitamins by molecular distillation. Nature, vol. 138, p. 881.

———— 1937 Molecular distillation; apparatus and methods. Ind. Eng. Chem., vol. 29, p. 968.

KOEHN, C. J., AND W. C. SHERMAN 1940 The determination of vitamin A and carotene with the photoelectric colorimeter. J. Biol. Chem., vol. 132, p. 527.

LOVERN, J. A., AND R. A. MORTON 1939 The distribution of vitamins A and A_2. III. Biochem. J., vol. 33, p. 330.

LOVERN, J. A., T. H. MEAD AND R. A. MORTON 1939 a Halibut intestinal oil. Biochem. J., vol. 33, p. 338.

MCLEAN, H., AND I. S. MCLEAN 1927 Lecithin and Allied Substances. 2nd ed. London. p. 80.

MOLL, T., AND A. REID 1939 On the different actions of vitamin A concentrates from fish liver oils. Z. Physiol. Chem., vol. 206, p. 9.

MORGAN, RONALD S., JOSEPH R. EDISBURY AND R. A. MORTON 1935 A discrepancy between the biological assays and other methods of determining vitamin A. I. Biochem. J., vol. 29, p. 1645.

SINCLAIR, GORDON R. 1928 Fat absorption: The influence on phospholipids of the small intestine. Ph.D. thesis, University of Rochester, p. 23.

EFFECT OF FLUORINE ON THE ACTIVITY OF VITAMIN D IN RACHITIC RATS

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FOUR FIGURES

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The toxicology and physiology of fluorine are being studied from two somewhat different points of view: (1) as a general protoplasmic poison and (2) as a modifier of calcium and phosphorus metabolism, the latter, chiefly through its action on bone and tooth development in growing animals. Quantitatively, a great deal is now known concerning the factors which influence the healing of rickets produced in rats on high calcium-low phosphorus diets such as the no. 2965 ration of Steenbock and Black ('25). This is especially true of the amounts of vitamin D required to produce definite degrees of healing or recalcification in the rachitic metaphysis. It occurred to us, therefore, that the standard vitamin D assay technique should prove a useful tool in the study of the effects of fluorine on those phases of calcium and phosphorus metabolism involving bone formation in young growing animals.

In the experiments described herewith, we have found definite radiographic evidence that fluorine does markedly affect both the development of rickets and the healing produced by vitamin D. Furthermore, the influence of this agent on the healing process appears to depend on both duration and degree of the toxicosis which exists at the time the vitamin is administered.

EXPERIMENTAL

Three experiments will be described. The first consists of observations made incidentally in connection with another study of fluorosis (Hodge and Finn, '39). The other two were designed specifically to test the effects of fluorine on vitamin D action. All observations were made by the radiographic technique of O'Brien and Morgareidge ('38), and in the two latter experiments the young rats were taken from our standardized vitamin D assay colony.

Experiment 1. One hundred and thirty-five stock rats, taken at weaning (21 days), were divided into three groups of forty-five each. Litter mates and sexes were distributed as equally as possible among the groups. The control group was fed a basal diet which has been shown to be adequate for normal growth and bone development (Hoppert, Webber and Canniff, '31) and which has the following composition:

	Per cent
Whole yellow corn (freshly ground)	58
Whole powdered milk	30
Linseed meal	6
Alfalfa leaf meal	3
Dried yeast [1]	2
Sodium chloride	1

[1] From the Northwestern Yeast Co., Chicago, Ill.

For the first experimental group, the whole powdered milk was replaced by an equal amount of commercial casein and will be referred to as the "casein diet." This diet failed to support normal growth or bone development. The second experimental group also received the casein diet but, in addition, was given 3.0 mg. of fluorine daily in 1 drop of a water solution of potassium fluoride delivered orally from a calibrated dropping pipette. Both basal and casein diets together with distilled water for drinking were provided ad libitum.

On the fiftieth day of this regime, radiograms of the left knee-joints were made. The control group (basal diet) was found to exhibit entirely normal bone structure (fig. 1 a).

The rats on the casein diet, however, showed evidence of rickets as indicated by the fact that about 50% of the animals had moderately decalcified metaphyseal zones (fig. 1 b). Although the rats receiving the casein diet plus fluorine also exhibited a similar rachitic picture as judged by the width and density of the uncalcified metaphyses, the tibial shafts and areas of the diaphysis proximal to the rachitic zones were, if anything, more densely calcified than normal (fig. 1 c).

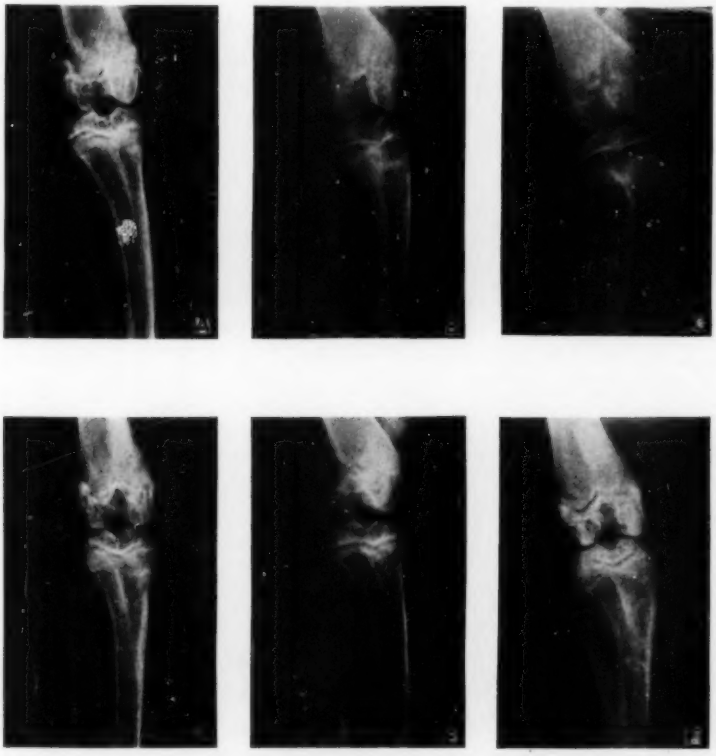

Fig. 1 A and D: Radiograms of same rat before and after addition of cod liver oil to control diet. B and E: Radiograms of same rat before and after addition of cod liver oil to the casein diet. C and F: Radiograms of same rat before and after addition of cod liver oil to the fluorine contaminated casein diet.

On the fifty-first day, 2% of cod liver oil was added to the diets of all three groups, replacing an equivalent amount of corn meal. Ten days later radiographic examination was repeated. The bones of the control group remained unchanged in appearance, i. e., they were normal (fig. 1 d). Obviously, the whole powdered milk had contained enough antirachitic activity to allow normal development. The rickets which had been observed in the animals on the casein diet had healed in a normal fashion as shown by a reduction in width of the uncalcified zone to that of the normal epiphyseal cartilage (fig. 1 e). In the group receiving the casein diet plus fluorine, however, the effect of the added vitamin D was the production of an atypical, unorganized calcification in which the epiphyseal cartilage was nearly obliterated (fig. 1 f). The effect of fluorine in this case, therefore, would appear to be that of disorganizing the bone-forming cells of the matrix without interfering markedly with the deposition of inorganic material under the stimulus of vitamin D.

Experiment 2. Three hundred parts per million of fluorine were incorporated into the Steenbock 2965 diet. This was done by thoroughly mixing a saturated solution of ammonium fluoride in 95% alcohol with the dry diet. The wet mixture was then spread in thin layers on large sheets of wrapping paper, allowing the alcohol to evaporate. Young rats, weaned at 21 days, were divided into two groups of twenty-six each. One group was placed on the fluorine contaminated 2965 diet and the other, on the same diet without the fluorine. After a rachitogenic period of 21 days, radiographs of the animals in each group were made and the type and degree of rickets compared. The results are seen in figure 2, in which the knee joints of three representative rats from the control group (top row) may be compared with those of three rats from the fluorine group (bottom row). The most obvious difference is the greater over-all density in the bones of the fluorine-fed animals. Also, the metaphyseal zone of hypertrophic cartilage is narrower in the fluorine group. Thus, as indicated by x-ray examination, a 300 p.p.m. contamination of

fluorine in the rachitogenic diet prevents the development of the usual rachitic picture.

In accordance with the usual assay procedure for vitamin D, international standard irradiated ergosterol solution, diluted in corn oil, was then administered to both groups of rats at a level of 0.5 I. U. per day for 8 days. On the tenth

Fig. 2 Top row: Radiograms of three representative rats from the control group after 21 days on the 2965 diet showing ordinary rickets. Bottom row: Radiograms of three representative rats from the group which had received the 2965 diet contaminated with 300 p.p.m. of fluorine for 21 days. Note the abnormal metaphyseal calcification and generally increased density in this "fluorine rickets."

day, another radiographic examination was made. Figure 3 (top row) shows the appearance of the healing obtained in three representative rats from the fluorine-fed group as contrasted with that obtained in the normally rachitic animals of the control group (bottom row). Again, the effect of fluorine is striking. The new calcification in the fluorine-fed rats is diffuse and appears to be continuous with the old calcification which was previously present in the diaphyseal

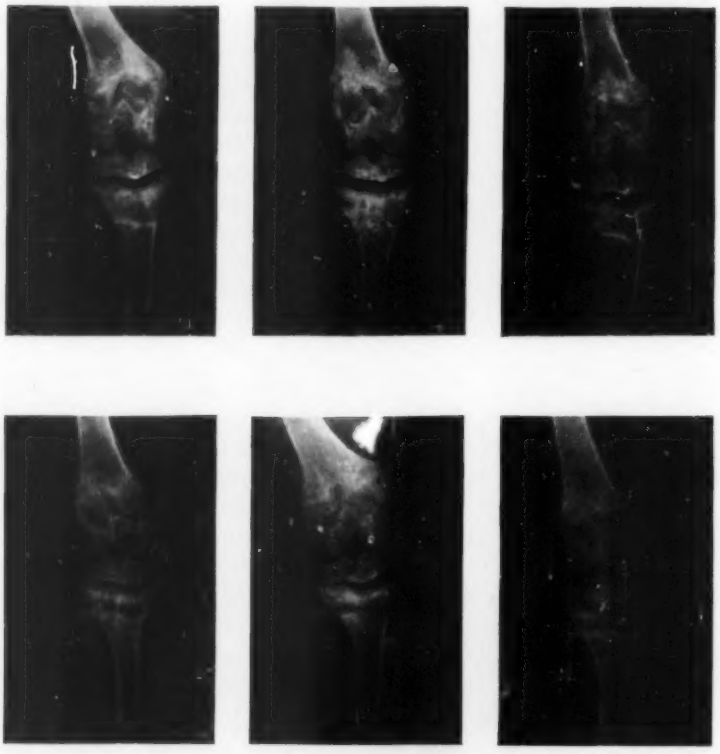

Fig. 3 Top row: Three rats showing type of healing produced by 0.5 I. U. of vitamin D per day for 8 days subsequent to production of rickets on the fluorine contaminated 2965 diet. Bottom row: Normal healing produced by the same dose of vitamin in the control group.

border. It would be extremely difficult, if not impossible, to make any quantitative estimation of vitamin D activity from such pictures. Thus, fluorine contamination of a diet from an accidental source, such as was recently reported (Hodge, Luce-Clausen and Brown, '39), would lead to severe distortion of vitamin D assay results.

Experiment 3. The results of the two preceding experiments suggested that some effect could be expected from the simultaneous administration of fluorine and vitamin D to rachitic rats which had had no previous exposure to this element. Accordingly, five groups of seven rats each were prepared for vitamin D assay by the usual 21-day period on the 2965 diet. Before starting the test feeding, radiographic examination was made to establish the fact that all had attained a uniformly satisfactory degree of rickets according to the standards set up for our routine assay work. The animals, at this time, were 42 days old and averaged between 50 and 55 gm. in weight. Each of the 35 rats was then given 0.5 I. U. of vitamin D (as the international standard solution) per day for 8 days. One group was kept for control and the other four given graded doses of fluorine in 1 drop of aqueous potassium fluoride solution, daily. The levels of fluorine fed were 0.5, 1.0, 2.0, and 3.0 mg., respectively, for the four groups. Final radiograms were made on the tenth day and the degree of healing found in the fluorine-fed animals compared with that of the controls. It was found that the curative power of the vitamin had been definitely inhibited by the fluorine and that the amount of inhibition was roughly proportional to the dose given. The healing produced by the half unit of vitamin D plus 3.0 mg. of fluorine was only 50% of that resulting from the same dose of vitamin alone. Similarly, the 0.5 mg. dose of fluorine resulted in about a 10% inhibition in healing.

The "line" of new calcification seen in these animals was quite typical of normal healing produced by vitamin D. The doses of fluorine used over the 10-day period of the experiment were not sufficient to produce symptoms of toxicity.

The diffuse, unorganized deposition of bone salt seen in the two previous experiments was absent. The radiograms in figure 4 (top row) show three representative rats from the control group which received 0.5 I. U. of vitamin per day with no fluorine. Figure 4 (bottom row) shows three rats from the group given 3.0 mg. of fluorine and the same dose of vitamin D. The marked reduction in healing caused by fluorine is evident.

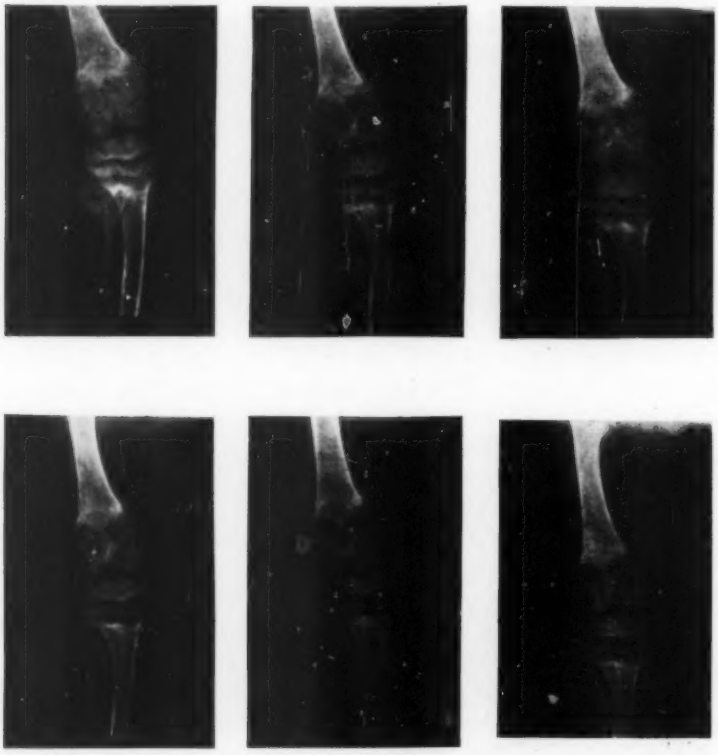

Fig. 4 Top row: Normal healing produced in rachitic rats by 0.5 I. U. of vitamin D per day for 8 days. Bottom row: Healing produced by the same amount of vitamin D administered simultaneously with 3.0 mg. of F per day. These animals were not exposed to fluorine during the rachitogenic period. Note the reduction in the amount of healing.

DISCUSSION

Hauck, Steenbock and Parsons ('33) added 0.15% fluorine to the 2965 diet and found an increase in total bone ash over that of control rats on the same diet without fluorine. Their experimental period was 6 weeks. When vitamin D was also incorporated into the fluorine-containing ration, they obtained a slightly higher ash content of the bone over that produced by vitamin D alone. Our radiographic evidence, especially in experiment 2, confirms their findings and shows, in addition, that the greatest concentration of this extra calcification accumulates near the diaphyseal border of the metaphysis. It is of interest to note that the authors quoted above also found that in diets with a normal Ca:P ratio, the addition of fluorine failed to increase the ash content of the bones.

Our experiment 3, however, indicates that a distinction must be made between the chronic and acute types of fluorosis as regards their effect on vitamin D. The fact that fluorine has the power to inhibit the classical picture of healing rickets suggests that further experiments of this type may shed some additional light on the mode of action of vitamin D.

SUMMARY

Summarizing, we have found: (1) In rats on a rachitogenic diet containing fluorine, there appears to be a decrease in the severity of the rickets due to a general increase in the density of the bones to x-rays and, more particularly, to a decrease in width of the metaphyseal zone of hypertrophic cartilage. (2) Vitamin D promotes the healing of this "fluorine rickets" but the deposition of new bone salt is atypical, its appearance suggesting that fluorine has in some way interfered with the orderly arrangement of the matrix cells. (3) Fluorine inhibits the healing process when given simultaneously with vitamin D to rachitic rats which have not been previously subjected to fluorine poisoning. This inhibition is roughly proportional to the dose given and amounts to 50% at a level of 3.0 mg. of fluorine per day.

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LITERATURE CITED

HAUCK, HAZEL M., H. STEENBOCK AND HELEN T. PARSONS 1933 The effect of the level of calcium intake on calcification of bones and teeth during fluorine toxicosis. Am. J. Physiol., vol. 103, p. 489.

HODGE, HAROLD C., AND SIDNEY B. FINN 1939 Reduction in experimental rat caries by fluorine. Proc. Soc. Exp. Biol. Med., vol. 42, p. 318.

HODGE, HAROLD C., ETHEL M. LUCE-CLAUSEN AND ELIZABETH F. BROWN 1939 Fluorosis in rats due to contamination with fluorine of commercial casein. The effects of darkness and controlled radiation on the pathology of the teeth. J. Nutrition, vol. 17, p. 333.

HOPPERT, C. A., P. A. WEBBER AND T. L. CANNIFF 1931 Production of dental caries in rats fed an adequate diet. Science, vol. 74, p. 77.

O'BRIEN, BRIAN, AND KENNETH MORGAREIDGE 1938 Radiographic demonstration of protection by vitamin D against metaphyseal decalcification in adult rats on high calcium—low phosphorus diet. J. Nutrition, vol. 16, p. 91.

STEENBOCK, H., AND A. BLACK 1925 Fat soluble vitamins. XXIII. The induction of growth promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. J. Biol. Chem., vol. 64, p. 263.

EDITORIAL REVIEW

MANGANESE, LEAD, TIN, ALUMINUM, COPPER, AND SILVER IN NORMAL BIOLOGICAL MATERIAL

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The spectrochemical data on manganese, lead, tin, aluminum, copper, and silver in the diet, the tissues, and the excreta of man given in a recent article (Kehoe et al., '40) were obtained primarily as a basis for our future investigations, but since, of necessity, they contradict, confirm, or supplement the observations of others it is our present purpose to review the more recent work and to summarize the pertinent facts concerning the normal occurrence of these metals in biological material.

MANGANESE

Manganese might be expected to occur generally in biological material, considering its recognized physiologic usefulness (Langecker, '34; von Oettingen, '35) and this expectation has been confirmed by many observations. Comparatively large quantities (approximately 4 mg. per day) are found in the human diet and substantially equivalent amounts are eliminated in the excreta (Kehoe et al., '40). The chief source of manganese in the diet, as shown by Boycott and Cameron ('30), Remington and Shiver ('30), Richards ('30), and Peterson and Skinner ('31), is of vegetable origin, cereals being particularly rich in this metal (Davidson, '29; Richards, '30).

The regular occurrence of manganese in the tissues of animals has been demonstrated among others by Bertrand and Ciurea ('31), Reiman and Minot ('20), Turnwald and Haurowitz ('29) and Chevalier ('30). The latter found low concentrations of manganese in the organs and noted that there was

not much variation in the average concentration in most tissues (0.03 to 0.04 mg. per 100 gm. of fresh tissue). Our data ('40) agree with these observations and particularly with those of Reiman and Minot ('20) who reported values of 0.17, 0.061, 0.035 and 0.036 mg. per 100 gm. of liver, kidney, brain, and stomach, respectively. Information on the distribution of manganese in the skeleton is scanty and our results of 0.17–0.30 mg. per 100 gm. of human bone ('40) are somewhat higher than the mean of 0.101 mg. per 100 gm. of the bones of rabbits, cats and dogs reported by Lund, Shaw and Drinker ('21).

Bertrand and Medigreceanu ('13), Reiman and Minot ('20), Abderhalden and Möller ('28), Dutoit and Zbinden ('29) and Adam and Horner ('37) found manganese in the blood of normal animals regularly. With the exception of one result of 1.15 mg. per 100 ml. in horse serum (Abderhalden and Möller, '28), low concentrations have been found in the blood. Bertrand and Medigreceanu ('13) found a higher concentration of manganese in the blood of the sheep than in that of any other animal investigated. Their value of 0.002 mg. per 100 ml. of human blood is considerably lower than the mean of 0.014 mg. per 100 ml. calculated from Reiman and Minot's data ('20) and our mean of 0.015 mg. per 100 ml. ('40). Practically all of the manganese in human blood is found in the cells (Kehoe et al., '40), but Bertrand and Medigreceanu ('13), in the case of sheep blood, found approximately two-thirds in the plasma. Our results revealed slight but definite variations in the manganese concentration of repeated samples from the same individual (Kehoe et al., '40), in contradiction of Reiman and Minot's ('20) observations that the level of concentration was constant for each individual.

McCrackan and Passamaneck ('26) failed to find manganese in the urine of normal individuals since it was not present in concentrations of 0.02 mg. or more per liter. Probst ('33) using flame spectra also was unable to detect manganese in the urine even in patients treated with psorimangan (a proprietary colloidal manganese preparation). On the other hand our data ('40) indicate that manganese is always present in amounts

somewhat less than 0.01 mg. per liter. Probst's ('33) failure to detect it may be attributed to the lower sensitivity of the flame as compared to the arc method of excitation.

COPPER

Like manganese, copper is physiologically important and is apparently a constituent of practically all plant and animal tissues (Eichholtz, '34; Gerlach, '34; Elvehjem, '35; Hahn and Fairman, '36; Eggleton, '38). Since the detection of copper in blood by Sarzeau (1830), the distribution of this metal in the animal organism has been the subject of exhaustive study, but there is still some disagreement as to its range of concentration in certain tissues. This is especially true in the case of blood, in which concentrations from 0.05 to 0.28 mg. per 100 ml. have been reported (Tompsett, '34 a). Tompsett's ('34 a) figures of 0.185 to 0.229 mg. per 100 ml. are considered too high by Elvehjem ('35), while Sachs and his co-workers ('35) obtained values of 0.141 and 0.132 by two different methods. Guillemet ('32) found 0.088 mg. per 100 ml. of beef blood, and Elvehjem and his collaborators ('29) approximately 0.05 mg. per 100 ml. of horse blood. The results of Schönheimer and Oshima ('29) (0.113 to 0.14 mg. per 100 ml. of human blood), and the average value of 0.098 reported by Bence and collaborators ('36), as well as the range of 0.091–0.108 given by Sarata ('35), are not far from our figure of 0.114 ('40). There is also some disagreement as to the distribution of copper between cells and plasma of blood. Bjerrum and Henriques ('35) found that the copper in rabbit blood was equally distributed between cells and plasma; Tompsett ('34 a) reported like results for human blood, but Guillemet ('32) found more copper in the plasma than in the cells of dog, pig, and ox blood. On the other hand, Elvehjem and co-workers ('29) reported that the cells of horse blood contained more copper than the plasma, and Sarata ('35) has given values of 0.034–0.064 mg. in plasma and 0.068–0.073 mg. in the erythrocytes. Our data ('40) generally confirm the latter figures but reveal the occurrence of exceptions to the rule that the concentration in the erythro-

cytes exceeds that in the plasma. The degree of variability in the copper content of the blood fractions from the same individual has not been determined, but it has been shown that the concentration in the whole blood is not constant (Kehoe et al., '40).

Our results on the copper concentration in human tissues ('40) are in general agreement with those on normal animal tissues reported by Bodansky ('21), Chou and Adolph ('35), Tompsett ('35), and Hahn and Fairman ('36). The relatively large quantities of copper found in the liver and brain are within the variations of 0.30 to 0.91 mg. per 100 gm. of liver and 0.22 to 0.46 mg. per 100 gm. of brain noted by Tompsett ('35), and 0.36 to 0.68 mg. per 100 gm. of brain found by Bodansky ('21). Comparatively large quantities of copper have also been found in bone. Tompsett ('35) gave results varying from 0.37 to 0.47 mg. per 100 gm. of rib, in conformity with our findings ('40). Tompsett did not analyze the long bones, and, except for our figure of 1.19 mg. per 100 gm. of such bones and for the report of Tiede and Chomse ('34) that copper is a natural constituent of teeth (1.2 mg. per 100 gm. of bovine teeth), we have not found additional data on bony structures.

The available data agree as to the daily ingestion and excretion of copper. The mean daily intake and output has been put at approximately 2 mg. per day by Chou and Adolph ('35), Tompsett ('35), Leverton ('39), and ourselves ('40). As in the case of manganese, most of the ingested copper is eliminated by the alimentary canal and only small quantities appear in the urine. Chou and Adolph ('35) report an average daily urinary output of 0.25 mg. per day, and Tompsett ('34 b) sets the average concentration at 0.18 mg. per liter. Leverton ('39) has recently reported variations in daily output among young women ranging from 0.025 to 0.325 mg., while Rabinowitch ('33) found concentrations varying from traces (ten instances) to 0.41 mg. per liter in the urine of fifty normal persons. Ross and Rabinowitch ('35) noted a similar range of variation in the urine of normal children. These results are considerably higher than ours ('40), which, for the most part, correspond

only to the lower range of the values given by Leverton ('39) and Rabinowitch ('33), a fact which raises the question of contamination in the case of the higher figures.

ALUMINUM

The effects of aluminum on the human organisms have been investigated extensively, and it is apparently established that the daily ingestion of amounts up to 200 mg. aluminum per day is harmless (U. S. Dept. Agr., '14; Burn, '32; Monier-Williams, '35). Sources of aluminum ingestion other than from cooking utensils and certain baking powders are found in foods of vegetable and animal origin as shown by Kahlenberg and Closs ('29), Underhill and Peterman ('29 b), Underhill et al. ('29), Yoshii and Jimbo ('32), Wührer ('33), Meunier ('36), and Webb ('37). Little of the ingested aluminum is absorbed, and in conformity with the findings of Underhill and Peterman ('29 a), Mackenzie ('30, '31), and Wührer ('33), our data ('40) show a practical equivalence between the amounts excreted daily in the feces and those occurring daily in the food.

The occurrence of aluminum in human tissues has been the subject of controversy (McCollum et al., '28, '30; Kahlenberg and Closs, '29). Our observations ('40) confirm the conclusions of the majority of investigators that aluminum is regularly present in small amounts. They are in excellent agreement with those of Myers and Mull on the normal tissues of man ('28 b), dog, and rat ('28 a), and with those of Wührer ('33) on the tissues of the dog.

Except for the results on two samples of dog blood given by Eveleth and Myers ('36), our mean concentration of 0.013 mg. per 100 ml. of blood (Kehoe et al., '40) is considerably lower than other recorded values. The values reported by Underhill and Peterman ('29 b) and by Mull and his co-workers ('27) approach 0.20 mg. per 100 ml. of blood. The latter investigators obtained lower values when larger samples were used, a fact highly suggestive of some factor of contamination. The carriage of almost all of the aluminum of the blood in the plasma and not in the formed elements, as shown by our

observations (Kehoe et al., '40), has apparently not been reported by others.

TIN

In agreement with our data ('40) tin has been found irregularly in normal biological material by a number of investigators. Newell and McCollum ('31) and Webb ('37) found tin in certain marine products while Boyd and De ('33) detected it in human tissues and in occasional samples of vegetable material. Staud ('36) found it in canned beer, and Dingle and Sheldon ('38) noted its occurrence in processed milk of human and other origin. Tin was present in very small quantities in about 80% of our samples ('40). When found in blood, practically all of the tin was in the cells (Kehoe et al., '40). It occurred in appreciable quantities in the food and feces of normal American subjects and, as in the case of the other metals referred to herein, there was an approximate balance between the amounts excreted in the feces and the quantities ingested with the food (Salant et al., '14; Kehoe et al., '40).

Several investigators have regarded tin as a regular constituent of certain biological materials. Bertrand and Ciurea ('31) found it in nearly all organs of the ox, the horse and the sheep, the quantities varying from 0.05 to 0.40 mg. per 100 gm. for all dry tissues except the skin, hair and tongue. According to Schübel ('34), the quantities found in the muscle and mucous membrane of the tongue were so high, relatively, as to have some specific functional significance, but no such role has been defined. Misk ('23) accepted tin as a normal constituent of human viscera, and believed that the human body contained as much tin as zinc. Dutoit and Zbinden ('30) also found tin in all organs, and especially abundantly in the brain, spleen, and thyroid. In disagreement with their results on the brain we encountered lower concentrations of tin in this tissue than in any other (Kehoe et al., '40). Jost ('34) and Lowater and Murray ('37) found tin in all teeth examined while Scott and McMillen ('36) reported its regular presence in spinal fluid.

SILVER

Only 10% of the samples of human tissue which we analyzed contained silver, and then in concentrations which were rarely greater than 0.01 mg. per 100 gm. ('40). Larger quantities were found only in feces and prepared food samples, these, no doubt, resulting from the use of silver tableware. The quantities found in the feces and food were smaller than those of the other metals studied (0.06–0.08 mg. per day) but in this case also the balance between the quantity eliminated daily in the feces and that ingested daily in the food was maintained (Kehoe et al., '40).

This same irregularity in the occurrence of silver in normal biological material was noted by Dutoit and Zbinden ('30), Fox and Ramage ('30, '31), Newell and McCollum ('31), Boyd and De ('33), Ramage, Sheldon and Sheldon ('33), Rusoff and Gaddum ('38), Kogan and Nasuirova ('35) and Lowater and Murray ('37).

LEAD

Our observations on the occurrence of lead in the tissues and excreta of normal human beings, up to 1933, together with a review of the results of other workers, have been detailed elsewhere (Kehoe et al., '33 a, '33 b, '33 d). Recently published data (Kehoe et al., '40) have provided further evidence of the correctness of our statement that "the presence of lead in human excreta and human tissues, as well as in living organisms in general, is an inevitable consequence of life on a lead bearing planet" (Kehoe et al., '33 a).

The freely chosen human diet varies considerably in its lead content from day to day (Kehoe et al., '40), and, as we have pointed out (Kehoe et al., '33 c), the increment of lead content above that which is present naturally is due to contamination from a great variety of sources. These sources have also been discussed by Monier-Williams ('38) whose data on the natural lead content of certain foods are in general agreement with our own. Observations on a series of subjects have shown that the mean daily lead intake in the diet is approximately

equivalent to the mean daily output of lead in the feces, it being about 0.30 mg. per day (Kehoe et al., '33 b, '35, '40).

The mean concentrations of lead in normal human tissues which we have reported (Kehoe et al., '33 b, '40) are in close agreement with those obtained in recent years by other investigators (Barth, '31; Weyrauch and Müller, '33; Lynch et al., '34; Tompsett and Anderson, '35; Tompsett, '36; Pernice, '36, and Bagchi et al., '39). The extent of the variation in the gross quantity of lead in the normal adult human body, and especially in the skeleton, is somewhat questionable. Barth's data ('31), in his opinion, pointed to a progressive increase in skeletal lead with age, and Calvery ('38) has made a similar interpretation of Tompsett's data ('36) on the lead content of the femur in a series of cases. Bagchi et al. ('39) did not find concentrations of lead in normal human bones in the higher range of Tompsett's values, nor have we obtained such results except in persons with definite histories of lead exposure. Our data up to the present do not demonstrate any relationship between lead concentration in the skeleton and age ('40).

All recent investigators are in agreement as to the normal occurrence of lead in human blood, the lowest values (with an upper limit at 0.04 mg. per 100 ml. of blood) having been given by Litzner and Weyrauch ('32, '33). Teisinger ('36) has set the mean concentration at 0.061 mg. per 100 ml., Tompsett and Anderson ('35) have given it as 0.055 mg., while Smith and co-workers ('38) found variations between 0.01 to 0.05 mg. per 100 cc. of normal blood. The data of Taeger and Schmitt ('37) derived from the analysis of the blood of 148 normal persons yield a mean concentration of 0.035 mg. per 100 cc. of blood, while Willoughby and Wilkins ('38) obtained a mean of 0.025 mg. per 100 cc. of blood in 189 normal cases. The mean value of our earlier results on seventy-one normal young men was 0.058 mg. per 100 gm. of blood (Kehoe et al., '35), but refinements in analytical technique and, more importantly,

rigid precautions in collecting and handling samples have reduced the mean of our results (Kehoe et al., '40) to a point which coincides with that of Willoughby and Wilkins, individual values ranging from 0.01 to 0.06 mg. per 100 gm. of whole blood.

In determinations of the distribution of lead in the blood fractions Willoughby and Wilkins ('38) obtained negative results in approximately 90% of fifty-eight samples of blood serum, and only insignificant quantities in the remaining samples. Smith and co-workers ('38) also failed to find lead in normal serum, while Teisinger ('36) found only minute quantities in the serum. The latter also studied the distribution between the plasma and cells and reported that the lead was equally divided between the two. This is in disagreement with our findings (Kehoe et al., '40) which clearly indicate that the normal plasma is almost free of lead. The results on twenty-seven samples of normal human plasma ranged from 0 to 20% of the total lead in the blood, twenty-three yielding less than 10%.

Schmitt and Basse ('38) studying the diurnal variations in the concentration of lead in the blood of five normal persons found elevated concentrations during the early morning hours. Our corresponding observations on the blood of normal persons (which with corresponding urinary findings will be reported elsewhere) have failed to show significant variations during the 24-hour period.

The mean values established for the lead concentration in normal urine (approximately 0.030 mg. per liter) (Kehoe et al., '35, '36, '40) are in general correspondence with the results of Bass ('33), and Behrens and Taeger ('35) in Germany, Ross and Lucas ('35) in Canada, Boyd and Ganguly ('32) in India, Reith and van Dijk ('38) in Holland and Tompsett and Anderson ('35) in England, if due allowance is made for variations in the sensitivity and accuracy of the analytical methods employed by these investigators.

LITERATURE CITED

ABDERHALDEN, E., AND P. MÖLLER 1928 The iron, copper and manganese content of blood serum. Z. physiol. Chem., vol. 176, p. 95.

ADAM, W. B., AND G. HORNER 1937 The tin content of English canned fruits and vegetables. J. Soc. Chem. Ind., vol. 56, p. 329.

BAGCHI, K. N., H. D. GANGULY AND J. N. SIRDAR 1939 Lead in human tissues. Indian J. Med. Research, vol. 26, p. 935.

BARTH, E. 1931 Lead content of human bones. Virchows Arch. f. path. Anat., vol. 281, p. 146.

BASS, E. 1933 Relations between clinical aspects of lead poinsoning and lead content of blood and urine. Deut. med. Wschr., vol. 59, p. 1665.

BEHRENS, B., AND H. TAEGER 1935 Quantitative determination of elimination of lead in urine of healthy persons and of persons with lead poisoning, by means of diphenylthiocarbazone. Ztschr. f. d. ges. exper. Med., vol. 96, p. 282.

BENCE, J., J. LENDVAI AND J. SZÉKELY 1936 Copper content of the blood in anemia. Z. klin. Med., vol. 130, p. 299.

BERTRAND, G., AND V. CIUREA 1931 Tin in the animal organism. Compt. rend., vol. 192, p. 780.

BERTRAND, G., AND F. MEDIGRECEANU 1913 The manganese normal of blood. Ann. Inst. Pasteur, vol. 26, p. 1013.

BJERRUM, J., AND V. HENRIQUES 1935 Determination of the copper content of plasma and red blood cells by the Warburg cysteine-oxidation method. Skand. Arch. Physiol., vol. 72, p. 271.

BODANSKY, M. 1921 The zinc and copper content of the human brain. J. Biol. Chem., vol. 48, p. 361.

BOYCOTT, A. E., AND G. R. CAMERON 1930 Manganese in foodstuffs. Lancet, vol. 2, p. 959.

BOYD, T. C., AND H. D. GANGULY 1932 Lead in urine. Indian J. Med. Research, vol. 20, p. 75.

BOYD, T. C., AND N. K. DE 1933 Some applications of the spectroscope in medical research. Indian J. Med. Research, vol. 20, p. 789.

BURN, J. H. 1932 The physiological action of aluminium. The Analyst, vol. 57, p. 428.

CALVERY, H. O. 1938 Chronic effects of ingested lead and arsenic: a review and correlation. J. Am. Med. Assoc., vol. 111, p. 1722.

CHEVALIER, G. 1930 Iron and manganese in the human organism. J. pharm. chim., (8), vol. 12, p. 480.

CHOU, T.-P., AND W. H. ADOLPH 1935 Copper metabolism in man. Biochem. J., vol. 29, p. 476.

DAVIDSON, JEHIEL 1929 Manganese in cereals and cereal mill products. Cereal Chem., vol. 6, p. 128.

DINGLE, H., AND J. H. SHELDON 1938 Spectrographic examination of the mineral content of human and other milk. Biochem. J., vol. 32, p. 1078.

DUTOIT, P., AND C. ZBINDEN 1929 Spectrographic analysis of the ash of blood and of organs. Compt. rend., vol. 188, p. 1628.

———— 1930 A spectrographic analysis of the ashes of human organs. Compt. rend., vol. 190, p. 172.

EGGLETON, W. G. E. 1938 Trace elements in nutrition. The Caduceus (Univ. of Hong Kong), vol. 17, p. 47.

EICHHOLTZ, F. 1934 Copper. Heffter-Heubner Handb. d. exptl. Pharmakol., vol. 3, pt. 3, pp. 1928–1971.

ELVEHJEM, C. A. 1935 The biological significance of copper and its relation to iron metabolism. Physiol. Rev., vol. 15, p. 471.

ELVEHJEM, C. A., H. STEENBOCK AND E. B. HART 1929 Is copper a constituent of the hemoglobin molecule? The distribution of copper in blood. J. Biol. Chem., vol. 83, p. 21.

EVELETH, D. F., AND V. C. MYERS 1936 Studies on aluminum. I. A critical study of quantitative colorimetric methods for aluminum on biological material. J. Biol. Chem., vol. 113, p. 449.

FOX, H. M., AND H. RAMAGE 1930 Spectrographic analysis of animal tissues. Nature, vol. 126, p. 682.

———— 1931 Spectrographic analysis of animal tissues. Proc. Roy. Soc. (London), vol. 108B, p. 157.

GERLACH, WERNER 1934 The copper content of human and animal organs. Virchows Arch. f. path. Anat., vol. 294, p. 171.

GUILLEMET, R. 1932 The distribution of copper among the different constituents of normal blood. Compt. rend., vol. 109, p. 652.

HAHN, P. F., AND E. FAIRMAN 1936 The copper content of some human and animal tissues. J. Biol. Chem., vol. 113, p. 161.

JOST, L. 1934 The occurrence of heavy metals in the teeth and salivary glands. Deut. Zahn., usw. Heilk., vol. 1. p. 117; Ber. ges. Physiol. u. exptl. Pharmakol., vol. 83, p. 494.

KAHLENBERG, L., AND J. O. CLOSS 1929 The presence of aluminum in animal and plant matter. J. Biol. Chem., vol. 83, p. 261.

KEHOE, R. A., F. THAMANN AND J. CHOLAK 1933 a On the normal absorption and excretion of lead. I. Lead absorption and excretion in primitive life. J. Indust. Hyg., vol. 15, p. 257.

———— 1933 b The same. II. Lead absorption and lead excretion in modern American life. Ibid., p. 273.

———— 1933 c The same. III. The sources of normal lead absorption. Ibid., p. 290.

———— 1933 d The same. IV. Lead absorption and excretion in infants and children. Ibid., p. 301.

———— 1935 Normal absorption and excretion of lead. J. Am. Med. Assoc., vol. 104, p. 90.

———— 1936 An appraisal of the lead hazards associated with the distribution and use of gasoline containing tetraethyl lead. II. The occupational lead exposure of filling station attendants and garage mechanics. J. Indust. Hyg. and Toxicol., vol. 18, p. 42.

KEHOE, R. A., J. CHOLAK AND R. V. STORY 1940 A spectrochemical study of the normal ranges of the concentration of certain trace metals in biological materials. J. Nutrition. (In press.)

KOGAN, A. M., AND K. M. NASUIROVA 1935 Investigation of food products of vegetable and animal origin for their copper content. Voprosui Pitaniya, vol. 4, p. 30.

LANGECKER, H. 1934 Manganese. Heffter-Heubner Handb. d. exptl. Pharmakol., vol. 3, pt. 2, pp. 1287–1400.

LEVERTON, R. M. 1939 The copper metabolism of young women. Proc., Am. Institute of Nutrition, J. Nutrition, vol. 17, suppl., p. 17.

LITZNER, S., AND F. WEYRAUCH 1932 Investigations of the lead content of the blood and urine, its relations to the appearance of clinical symptoms and its diagnostic significance. Arch. f. Gewerbepath. u. Gewerbehyg., vol. 4, p. 74.

———— 1933 Value of determining lead content of blood in medico-legal judgment of injuries due to lead poisoning. Med. Klin., vol. 29, p. 381.

LOWATER, F., AND M. M. MURRAY 1937 Chemical composition of teeth. F. Spectrographic analysis. Biochem. J., vol. 31, p. 837.

LUND, C. C., L. A. SHAW AND C. K. DRINKER 1921 Quantitative distribution of particulate material (manganese dioxide) administered intravenously to the dog, rabbit, guinea pig, rat, chicken, and turtle. J. Exp. Med., vol. 33, p. 231.

LYNCH, G. R., R. H. SLATER AND T. G. OSLER 1934 Determination of traces of lead in biological materials, with special reference to bone. The Analyst, vol. 59, p. 787.

MACKENZIE, K. 1930 Biochemistry of aluminum. I. Excretion and absorption of aluminum in the pig. Biochem. J., vol. 24, p. 1433.

———— 1931 The same. II. Excretion and absorption of aluminum in the rat. Ibid., vol. 25, p. 287.

MCCOLLUM, E. V., O. S. RASK AND J. E. BECKER 1928 The possible role of aluminum compounds in animal and plant physiology. J. Biol. Chem., vol. 77, p. 753.

———— 1930 Do the spectrograms of Kahlenberg and Closs demonstrate the presence of aluminum in biological matter? Ibid., vol. 85, p. 779.

MCCRACKAN, R. F., AND E. PASSAMANECK 1926 Manganese in urine. Arch. Path. Lab. Med., vol. 1, p. 585.

MEUNIER, P. 1936 The presence and distribution of aluminum in animal tissues. Compt. rend., vol. 203, p. 891.

MISK, E. 1923 Tin in the human organism. Compt. rend., vol. 176, p. 138.

MONIER-WILLIAMS, G. W. 1935 Aluminum in food. Repts. Publ. Health and Med. Subjects No. 78.

———— 1938 Lead in food. Ibid., No. 88.

MULL, J. W., D. B. MORRISON AND V. C. MYERS 1927 The presence of aluminum in blood. Proc. Soc. Exptl. Biol. Med., vol. 24, p. 476.

MYERS, V. C., AND J. W. MULL 1928 a The influence of the administration of aluminum upon the aluminum content of the tissues and upon the growth and reproduction of rats. J. Biol. Chem., vol. 78, p. 605.

———— 1928 b The aluminum content of human autopsy tissue. Ibid., vol. 78, p. 625.

NEWELL, J. M., AND E. V. MCCOLLUM 1931 Spectrographic analysis of marine products. U. S. Dept. Commerce, Bureau of Fisheries, Inves. Rept. No. 5.

PERNICE, H. 1936 The liver in lead poisoning and in combination poisonings. Arch. f. Gewerbepath. u. Gewerbehyg., vol. 7, p. 538.

PETERSON, W. H., AND J. T. SKINNER 1931 Distribution of manganese in food. J. Nutrition, vol. 4, p. 419.

PROBST, R. 1933 Spectroanalytic demonstration of bismuth in tissues; determination of mercury in urine. Arch. exptl. Path. u. Pharmakol., vol. 169, p. 119.

RABINOWITCH, I. M. 1933 The copper content of urine of normal individuals. J. Biol. Chem., vol. 100, p. 479.

RAMAGE, H., J. H. SHELDON AND W. SHELDON 1933 Spectrographic investigation of the metallic content of the liver in childhood. Proc. Roy. Soc. (London), vol. 113B, p. 308.

REIMAN, C. K., AND A. S. MINOT 1920 A method for manganese quantitation in biological material together with data on the manganese content of human blood and tissues. J. Biol. Chem., vol. 42, p. 329.

REITH, J. F., AND C. P. VAN DIJK 1938 Microdetermination of lead in urine. Chem. Weekblad, vol. 35, p. 671.

REMINGTON, R. E., AND H. E. SHIVER 1930 Iron, copper and manganese content of some common vegetable foods. J. Assoc. Off. Agr. Chem., vol. 13, p. 129.

RICHARDS, M. B. 1930 Manganese in relation to nutrition. Biochem. J., vol. 24, p. 1572.

ROSS, J. R., AND C. C. LUCAS 1935 A new method for the determination of minute amounts of lead in urine. J. Biol. Chem., vol. 111, p. 285.

ROSS, A., AND I. M. RABINOWITCH 1935 The copper content of urine of normal children. J. Biol. Chem., vol. 111, p. 803.

RUSOFF, L. L., AND L. W. GADDUM 1938 The trace-element content of the newborn rat as determined spectrographically. J. Nutrition, vol. 15, p. 169.

SACHS, A., V. E. LEVINE AND A. A. FABIAN 1935 Copper and iron in human blood. Arch. Int. Med., vol. 55, p. 227.

SALANT, W., J. B. RIEGER AND E. L. P. TREUTHARDT 1914 Absorption and fate of tin in the body. J. Biol. Chem., vol. 17, p. 265.

SARATA, U. 1935 The biochemistry of copper. VI. Copper in relation to menstruation and pregnancy, with the copper content of men's blood. Japan. J. Med. Sci. II. Biochem., vol. 3, p. 1.

SARZEAU 1830 On the presence of copper in vegetable matter and in blood. J. Pharmacie, vol. 16, p. 505.

SCHMITT, F., AND W. BASSE 1938 Diurnal variation of the mineral movement between plasma and erythrocytes seen in the lead, calcium and phosphorus of healthy persons under various research conditions; lead content of the bile. Deut. Arch. f. klin. Med., vol. 182, p. 193.

SCHÖNHEIMER, R., AND F. OSHIMA 1929 The copper content of normal and pathological organs. I. Method. Z. physiol. Chem., vol. 180, p. 249.

SCHÜBEL, K. 1934 Tin. Heffter-Heubner Handb. d. exptl. Pharmakol., vol. 3, pt. 3, pp. 1560–1574.

SCOTT, G. H., AND J. H. MCMILLEN 1936 Spectrographic analyses of human spinal fluid. Proc. Soc. Exptl. Biol. Med., vol. 35, p. 287.

SMITH, F. L. 2ND, T. E. RATHMELL AND G. E. MARCIL 1938 Early diagnosis of acute and latent plumbism. Am. J. Clin. Path., vol. 8, p. 471.

STAUD, A. H. 1936 Using the spectrograph to detect metal contamination. Glass Packer, vol. 15, p. 731.

TAEGER, H., AND F. SCHMITT 1937 Quantitative determination by diphenylthiocarbazone of the lead content of blood and feces in healthy persons and in cases of lead poisoning. Ztschr. f. d. ges. exper. Med., vol. 100, p. 717.

TEISINGER, J. 1936 Rapid micropolarographic method for the quantitative determination of lead in the blood. Ztschr. f. d. ges. exper. Med., vol. 98, p. 520.

TIEDE, E., AND H. CHOMSE 1934 The copper content of teeth. Ber., vol. 67B, p. 1992.

TOMPSETT, S. L. 1934 a The copper content of blood. Biochem. J., vol. 28, p. 1544.

———— 1934 b Excretion of copper in urine and feces and its relation to the copper content of the diet. Ibid., vol. 28, p. 2088.

———— 1935 The copper and "inorganic" iron contents of human tissues. Ibid., vol. 29, p. 480.

———— 1936 The distribution of lead in human bones. Ibid., vol. 30, p. 345.

TOMPSETT, S. L., AND A. B. ANDERSON 1935 The lead content of human tissues and excreta. Ibid., vol. 29, p. 1851.

TURNWALD, H., AND F. HAUROWITZ 1929 The heavy metals of the human liver and their spectrographic detection. Z. physiol. Chem., vol. 181, p. 176.

UNDERHILL, F. P., AND F. I. PETERMAN 1929 a The metabolism of aluminum. III. Absorption and excretion of aluminum in normal man. Am. J. Physiol., vol. 90, p. 40.

———— 1929 b The same. II. Absorption and deposition of aluminum in the dog. Ibid., vol. 90, p. 15.

UNDERHILL, F. P., F. I. PETERMAN, E. G. GROSS AND A. C. KRAUSE 1929 The same. VII. Aluminum content of some fresh foods. Ibid., vol. 90, p. 72.

U. S. DEPARTMENT OF AGRICULTURE BULLETIN No. 103 (1914).

VON OETTINGEN, W. F. 1935 Manganese: its distribution, pharmacology and health hazards. Physiol. Rev., vol. 15, p. 175.

WEBB, D. A. 1937 Studies on the ultimate composition of biological material. II. Spectrographic analyses of marine invertebrates, with special reference to the chemical composition of their environment. Sci. Proc. Roy. Soc. (Dublin), vol. 21, p. 505.

WEYRAUCH, F., AND H. MÜLLER 1933 So-called normal lead in the human body. Ztschr. f. Hyg. u. Infekt., vol. 115, p. 216.

WILLOUGHBY, C. E., AND E. S. WILKINS 1938 The lead content of human blood. J. Biol. Chem., vol. 124, p. 639.

WÜHRER, J. 1933 The absorption of aluminum compounds in the organism and a consideration of the normal aluminum content of animal tissues. Biochem. Z., vol. 265, p. 169.

YOSHII, Y., AND T. JIMBO 1932 Microchemical detection of aluminum and its occurrence in the plant kingdom. Science Repts. Tôhoku Imp. Univ., Fourth Ser., vol. 7, p. 65.